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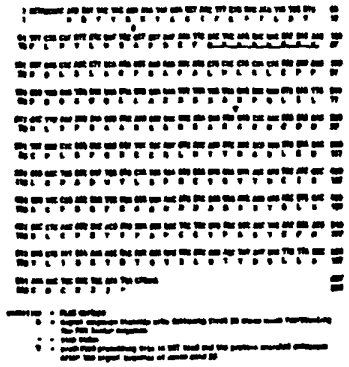
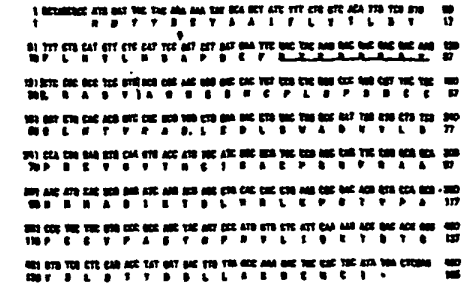
(54) Title: NOVEL TGF-β LIKE CYTOKINE

(57) Abstract

A novel TGF-β like cytokine is described which has been designated pCL13. Polynucleotide molecules encoding pCL13 and biologically active fragments are also described as well as methods of expression and uses of the proteins, fragments and polynucleotide molecules.





family members. The β1-LAP is cleaved from the mature protein, but remains disulphide bonded to it. Separation of the β1-LAP is necessary to achieve biological activity (2).

The TGF-β proteins have been studied intensively because of their biological importance and therapeutic potential. Their biology and functions are well known and have been extensively reviewed (e.g. 2, 3, 4). In general terms they promote differentiation and differentiated function in a wide variety of cells. They are potent chemotactic factors for macrophages and fibroblasts and generally inhibit cell proliferation, perhaps because of their role in differentiation. In the context of inflammation, TGF-β is a potent stimulator of fibroblast collagen and matrix protein synthesis, promotes angiogenesis, modulates expression of adhesion molecules and inhibits lymphocyte proliferation, production of some lymphokines and NK cell function. This molecule has been of great interest to the pharmaceutical industry mainly, because of its demonstrable capacity to promote wound and fracture healing *in vivo*. TGF-β has also been heavily implicated in the pathogenesis of chronic inflammatory processes and mechanisms. Further, its local production has been used as a surrogate marker e.g. in active fibrotic diseases such as cirrhosis and it therefore has potential in the diagnostic arena.

The present inventors have now isolated a polynucleotide molecule including a novel cytokine gene, clone 13 (CL13), which encodes a dimeric protein (pCL13) that appears to represent the first member of a new class of protein within the TGF-β superfamily.

Thus, in a first aspect, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding, or complementary to a sequence encoding, a protein designated pCL13 or a biologically active fragment thereof.

The isolated polynucleotide molecule may comprise a nucleotide sequence the same as that of the CL13 clone described herein or may contain single or multiple nucleotide substitutions and/or deletions and/or additions thereto. The nucleotide substitutions which are envisaged may result in one or more conservative or non-conservative amino acid substitution(s). By conservative substitutions, the intended combinations are - G,A; V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,Nα-alkylamino acids. The term "nucleotide sequence" also includes sequences with sufficient homology to

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NOVEL TGF-β LIKE CYTOKINE

This invention relates to a novel TGF-β like cytokine and to isolated polynucleotide molecules encoding this protein. Particular applications of the invention may include treatments for wound and fracture healing, treatments and diagnostic assays for cancer, autoimmune and fibrotic diseases.

Macrophages play a central role in chronic inflammatory processes. The importance of these cells derives from the large variety of bioactive molecules that they produce and consequently, their capacity to amplify the inflammatory response. Their central role is also due to their capacity for communication with many other cells. For example, macropage derived platelet derived growth factor (PDGF) is an important growth factor for both fibroblasts and smooth muscle cells. Another group of proteins of great significance in the relationship of macrophages with various connective tissue cells (e.g. fibroblasts, smooth muscle, endothelium osteoblasts etc) are the TGF-β superfamily cytokines, especially the TGF-β proteins themselves.

The TGF-β superfamily consists of growth and differentiation factors that share substantial structural homology (1). In vertebrates, individual families comprise the TGF-β proteins themselves, the growth and differentiation factors (GDF)(embryonic growth and development), the bone morphogenetic proteins (BMP)(induce cartilage and bone formation), the inhibins and activins (regulate FSH secretion by pituitary), and mullerian inhibitory substance (MIS)(regression of Mullerian duct during male sex differentiation). These proteins share important structural features. Their bioactivity resides in the carboxyterminal region of 100-150 amino acids. Over this region, members of this superfamily share about 30% sequence identity to TGF-β1 and have 7 conserved cysteine residues. Within individual subgroups of the superfamily, proteins share 70% to 90% identity over the bioactive carboxy terminal domain. All superfamily members are thought to be cleaved at a cluster of basic residues 110 to 140 amino acids from the carboxy terminus of a precursor protein. Processing occurs immediately following a conserved RXXR sequence.

The three human TGF-β proteins share 80% sequence similarity over the bioactive portion of the molecule. The pro peptide (called latency-associated peptide (β1-LAP)) displays less than 50% similarity between

cell lines, or may be produced recombinantly by any of the methods common in the art (5).

In a third aspect, the present invention provides an organism transformed with the polynucleotide molecule of the first aspect of the present invention.

The organisms which may be usefully transformed with the polynucleotide molecule of the first aspect include bacteria such as *E.coli* and *B.subtilis*, eukaryotic cell lines such as CHO, fungi, yeast, non-human animals and plants.

Transformed or transgenic, non-human animals may be established to, for example, overexpress CL13, pCL13 or a biologically active fragment thereof or, alternatively, generate antisense or ribozyme RNA molecules to inhibit native CL13 expression.

In a fourth aspect, the invention provides an antibody or fragment thereof specific to pCL13 or an antigenic portion thereof. The antibody may be polyclonal or monoclonal and may be produced by any of the methods common in the art.

It is also to be understood that the invention relates to kits for diagnostic assays, said kits comprising an antibody according to the fourth aspect of the present invention or nucleotide primers for PCR based assays.

In a fifth aspect the invention provides a protein or antigenic portion thereof, capable of binding to an anti-pCL13 antibody.

pCL13 is suitable for *in vivo* and *in vitro* procedures involving both human and animal cells. pCL13 is also suitable for both medical and veterinary use. In particular, pCL13 may be suitable for methods of treatment for any disease or condition beneficially treatable with TGF-β or another member of the TGF-β superfamily.

In a further aspect, the present invention provides a method of treatment to assist wound and/or fracture healing and/or ischaemic injury, comprising administering (for example, orally, topically, intravenously or subcutaneously) to a subject a preparation comprising a protein, or biologically active fragment thereof, according to the second or fifth aspects of the present invention, optionally in admixture with a suitable pharmaceutically acceptable carrier.

The protein, or biologically active fragment thereof, according to the second or fifth aspects may also be useful for one or more of the following:

hybridise with the nucleotide sequence under medium or, more preferably, high stringency conditions (5) and to nucleotide sequences encoding functionally equivalent sequences. In addition, the term "nucleotide sequence" includes sequences having at least 70%, more preferably 90%, homology to clone 13 described herein or any portion thereof of > 10 nucleotides in length.

Most preferably, the isolated polynucleotide molecule comprises a nucleotide sequence substantially corresponding to the nucleotide sequence shown in Figure 1 or a portion thereof, or a complementary sequence thereto. The term "portion thereof", in this regard, is to be understood as referring to portions of the nucleotide sequence which encode biologically active protein fragments and also, to portions of the nucleotide sequence, preferably >10 nucleotides in length, which may be used in, or for the production of probes useful for, hybridisation assays.

The present invention also further extends to oligonucleotide primers for the above sequences, antisense sequences and homologues of said primers and antisense sequences, complimentary ribozyme sequences, catalytic antibody binding sites and dominant negative mutants of the polynucleotide molecule.

In a second aspect, the invention provides a protein designated pCL13, or a biologically active fragment thereof, in substantially pure form.

Preferably, the protein, or biologically active fragment thereof, comprises a monomeric polypeptide having an amino acid sequence substantially corresponding to the amino acid sequence shown in Figure 1 or a fragment thereof.

Biologically active fragments thereof as mentioned in the first and second aspects refers to monomeric pCL13 polypeptides (with or without the propeptide) and other polypeptide or peptide portions (whether monomeric or dimeric) thereof which may consist of sequences which inhibit, mimic or enhance the biological effect of the protein and dominant negative protein mutants, binding proteins including soluble receptors, other protein and/or glycosaminoglycans. The pCL13 propeptide may also represent a biologically active fragment of pCL13.

The protein, or biologically active fragment thereof, according to the second aspect may be purified from natural sources (e.g. lungs, skin etc) or

The invention thus further resides in a heterodimeric protein comprising a monomeric polypeptide of pCL13 together with a monomeric polypeptide of another protein from the TGF-β superfamily.

pCL13 or biologically active fragments thereof may be formulated into standard pharmaceutical compositions suitable for the administration of proteins. Suitable formulations can be found, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA.

The dosage levels of pCL13 or biologically active fragments thereof may be comparable to those useful for other members of the TGF-β superfamily. These levels are well understood in the art, and the precise dosage can be adjusted according to the condition of the subject, the mode of administration, and the judgement of the attending physician.

Possible diagnostic applications include diagnosis of cancer, inflammatory and fibrotic disorders such as rheumatoid arthritis, cirrhosis and altherosclerosis in which enhanced synthesis of this gene may be present.

To facilitate the abovementioned applications for pCL13, it will be necessary to produce the protein in large quantities. However, extensive studies with other protein members of the TGF-β superfamily, has revealed a number of difficulties in achieving expression in commercial amounts. For instance, expression in simple prokaryotic systems are largely unsuitable since members of the superfamily are cysteine knot dimeric proteins having a complex pattern of disulphide bond linkages.

The current strategy for expression of TGF-β superfamily proteins is therefore to express the whole protein from a suitable DNA construct transfected into mammalian cells. However, this strategy necessitates treatment of the culture supernatant to separate processed (cleaved) bioactive mature protein from the propeptide and unprocessed (uncleaved) material. This creates additional costs and difficulties because some of the expressed material is non-productive as typically 30%-50% of the secreted material will not be appropriately cleaved. The additional chromatographic procedure also generates extra losses of protein and incurs additional cost and time.

(i) Immunosuppression and anti-inflammatory effects for conditions such as autoimmune diseases or transplantation;

(ii) Down regulation of leukocyte extravasation and motility in infective or inflammatory processes; and

(iii) Treatment of tumours through promotion of differentiation and antiproliferation action.

Such uses may be achieved by administration of the protein, or a biologically active fragment thereof, to a subject, or by gene therapy using all or part of the polynucleotide molecule of the first aspect. Such gene therapy may be used to, for example, establish overexpression of CL13, or pCL13 or a biologically active fragment thereof in the host cell or, alternatively, to generate antisense or ribozyme RNA molecules to inhibit native CL13 expression.

It is also possible that inhibiting the action of pCL13 may provide treatment of fibrotic/fibroproliferative disorders such as rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, scleroderma, liver cirrhosis and keloids, and inhibition of tumour immunosuppression associated with conditions such as tumours, infections (especially viral) and chronic inflammatory diseases. These treatments may be achieved by using:

fragments or peptides of the pCL13 protein that inhibit receptor binding;

binding proteins for pCL13 including soluble receptors for this molecule, glycosaminoglycans, and other molecules which may inhibit or destabilise receptor ligand interaction;

antibodies directed at pCL13 or its receptor;

antisense or ribozyme strategies in which expression or stability of the pCL13 gene product is disturbed;

dominant negative mutants of the CL13 gene which, when expressed in a host cell, will destabilise or affect the activity of pCL13. (As the pCL13 protein is a dimer, a second gene product which has been modified may bind to the native pCL13 to form a heterodimer). Thus, an appropriately modified pCL13 variant may essentially render the pCL13 inactive through mechanisms such as enhanced degradation, aberrant intracellular trafficking and inhibition of export from the cell and inhibition of bioactivity.)

Previous efforts to express the mature bioactive portion of these proteins alone, has been unsuccessful, indicating that the propeptide is essential for achieving expression and secretion.

The present inventors, however, have been unexpectedly able to achieve expression and secretion of pCL13 without expressing the leader or propeptide, using transfected mammalian cell cultures.

Thus, in a still further aspect, the present invention provides a method for producing a protein designated pCL13 or a biologically active fragment thereof, comprising transforming a suitable host organism with a polynucleotide molecule comprising a nucleotide sequence encoding pCL13 or a biologically active fragment thereof, wherein said polynucleotide molecule is constitutively or inducibly expressed in said host organism.

Preferably, the nucleotide sequence encoding the pCL13 or a biologically active fragment thereof, does not comprise sequence encoding the leader or propeptide of pCL13.

In place of sequences encoding the native leader or propeptide, it may be preferable to include within the polynucleotide molecule sequences encoding a heterologous leader (e.g. the follicle stimulating hormone (FSH) leader sequence) to assist expression.

Suitable host organisms may be any of those mentioned above in respect to the third aspect of the present invention. However, preferred organisms include mammalian cell lines, yeast (e.g. Pichia and Saccharomyces) and non-human animals.

Expression of only the mature bioactive portion of pCL13 thereby provides the following advantages:

(i) Higher levels of expression; and

(ii) No necessity to purify from propeptide and unprocessed full length CL13 protein.

Further, since it is not necessary to express the whole protein, it is possible and simple to add amino-terminal epitope tags (e.g. FLAG and/or HIS) that can significantly assist with the purification and visualisation of recombinant protein.

Also, the capacity to express the mature bioactive portion of pCL13 in mammalian cells, indicates that it will also be able to be readily expressed in yeast strains, such as the *Pichia pastoris* which is capable of secreting

disulphide linked proteins. Production of protein in yeast is much cheaper and easier than production by mammalian cells.

The invention will now be further described by way of the following non-limiting examples and with reference to the accompanying figures.

Brief Description of the Figures

Figure 1 provides the nucleotide sequence and putative amino acid sequence of clone 13 encoding pCL13.

Figure 2 shows CL13 expression in macrophage cultures.

Panel A. 15 μg total RNA was loaded per lane. Macrophage treatments were: lane 1, no treatment; lane 2, 1,000 U IFNγ overnight, lane 3, 1 μM retinoic acid overnight; lane 4, 1 μM retinoic acid overnight followed by 10 μg/mL LPS for 3 hours; lane 5, 10 μg/mL LPS for 3 hours.

Panel B. 20 μg total RNA was loaded per lane. Macrophage treatment were: lane 1, 1 μM retinoic acid for 3 days followed by 10 μg/ml LPS for 3 hours; lane 2, 1 μM retinoic acid overnight followed by 50 nM PMA for 3 hours; lane 3, 50 nM PMA for 3 hours; lane 4, untreated macrophages.

Figure 3 shows a northern blot analysis of clone 13 expression from macrophages treated with cytokines. All treatments were 3 hours. Lane 1, untreated macrophages; lane 2, 50 nM PMA; lane 3, 50 U/mL GM-CSF; lane 4, 100 U/mL M-CSF; lane 5, 100 U/mL IL1-β; lane 6, 10ng/mL TGF-β; lane 7, 10 U/mL PDGF-BB; lane 8, 50 U/mL IL-2; lane 9, 100 U/mL TNF-α, lane 10, 50 U/ml IL-6.

Figure 4 shows a northern blot analysis of the expression of clone 13 in U937. 20 μg total RNA was loaded per lane on a 1.2% agarose denaturing formaldehyde gel. Lane 1, no treatment; lane 2, 1 μM retinoic acid for 3 days, lanes 3, 4, 5, 6, 7, 1 μM retinoic acid for 3 days followed by 160 nM PMA for 20 min, 1 h, 2 h, 3 h and 12 h respectively; lane 8, 160 nM PMA for 3 h. Probes were labelled with ^{32}P. The blot was hybridized at 65°C and subjected to post hybridization washes and autoradiography.

Figure 5 provides a multiple sequence alignment of the carboxy terminal halves of pCL13 and other TGF-β superfamily members.

Figure 6 provides the nucleotide sequence and putative amino acid sequence for clone 13 in construct C13LB. The coding region for the bioactive portion of pCL13 commences with nucleotide 625.

Figure 7 provides the nucleotide sequence and putative amino acid sequence for clone 13 in construct FFC13S. The predicted bioactive portion of pCL13 commences with amino acid 92.

Figure 8 provides the nucleotide sequences and putative amino acid sequence for clone 13 in construct C13SA. The coding region for the bioactive portion of pCL13 commences with nucleotide 136.

Figure 9 shows a Western blot of purified recombinant pCL13 (FFC13S construct) visualised with anti-FLAG antibody.

Figure 10 provides a graph of the results obtained from glycosaminoglycan analysis in non-transfected (K1) and CL13 (FFC13S construct) transfected (P4N, 15 and 24) CHO cells using dimethyl-methylene blue (DMB) assay.

Figure 11 provides a graph of results obtained from collagen production assays of non-transfected (K1) and CL13 (FFC13S construct) transfected (P4N, 15 and 24) CHO cells.

Figure 12 provides graphs of results obtained from glycosaminoglycan production analysis in 3T3 (Figure 12A) and CCD (Figure 12B) cells following addition of various concentrations of pCL13 (expressed from construct C13SA).

Figure 13 provides graphical results obtained from collagen production analysis in CCD cells following addition of various concentrations of pCL13 (expressed from construct C13SA).

Figure 14 provides graphs of results showing growth factor activity under limiting serum conditions of pCL13 (expressed from construct C13SA) against TGFβ in human baby foreskin fibroblasts (BFF) (Figure 14A) and 3T3 cells (Figure 14B).

Figure 15 provides graphs of results showing growth factor activity in the presence of serum of pCL13 (expressed from construct C13SA) and TGFb in BFF and 3T3 cells.

Figure 16 provides graphs of results showing the effect of pCL13 (expressed from construct C13SA) of pCL13, TGFβ and IFNa2b on the proliferation of U937 human monocytic cells (Figure 16A) and mono Mac 6 human monocytic cells (Figure 16B).

Figure 17 provides graphical results of an analysis of differing pCL13 (expressed from construct C13SA) concentrations on TNF-α production in human culture derived macrophages.

Figure 18 provides graphs of results showing the effect of pCL13 (expressed from construct C13SA) concentrations on the cytoxicity of monocytes towards 5637 bladder tumour target cells (Figure 18A) and MDA-MB-231 breast tumour target cells (Figure 18B).

Figure 19A provides a micrograph of subcutaneous tissue taken from a rat having been administered pCL13 (expressed from construct C13SA).

Figure 19B provides a micrograph of subcutaneous tissue taken from a control rat having been administered saline only.

Figure 20A shows the nucleotide sequences of CL13 variants (a1, b1, b2, d2, dd2, f1, u2 and h1) and the original CL13 (denoted C13).

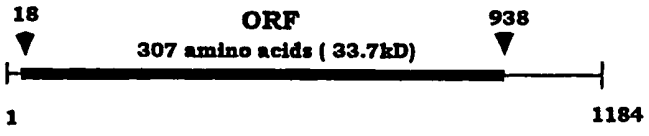
Figure 20B shows a comparison of a portion of the putative amino acid sequence of the CL13 variants a1, b1, b2, d2, dd2, f1, u2 and h1.

Figure 21 provides the nucleotide sequence and putative amino acid sequence for clone 13 in construct C13SA/5H (HIS Thrombin cleavage site-FLAG-PKA-mature bioactive CL13 peptide). This construct has been used for expression in the yeast *Pichia pastoris*. HIS is 5 histadine residue motif to allow affinity purification using Nickel chelate chromatography. The thrombin site is to allow enzymic cleavage of the HIS from the rest of the sequence if required.

Figure 22 shows a western blot of culture medium from *Pichia pastoris* transformed with construct C13SA/5H. pCL13 protein is visualised using anti-FLAG antibody.

EXAMPLE 1: Characterisation of Clone 13

This clone hybridizes on Northern blot to a single species of mRNA of 1.2kb size and the gene has been localised by fluorescent *in situ* hybridisation to chromosome 19p13.1 (TGF-b1 is on 19q13.1 and MIS is on 19p13.3). The characteristics of the clone are outlined below.



The largest open reading frame codes for a high cysteine containing protein with a signal peptide. It bears strong homology to members of the

TGF-β superfamily (including TGF-b itself) when analysed using the *fasta* program on the ANGIS facility (opt scores 180-250). Extensive multiple sequence alignment using the CLUSTAL V program on GCG has been undertaken with the CL13 translated amino acid sequence (pCL13) and most members of this superfamily (see Figure 2).

Mature pCL13 is a dimeric protein with a conserved RXXR site that is likely to be involved in cleavage of a large pro-peptide with the encoded polypeptide decreasing from a predicted monomeric mass of about 34kDa to 13kDa. pCl13 has a potential glycosylation sites in its pro-peptide, but none in the mature protein, suggesting that glycosylation may be for intracellular targeting as it is in TGF-β.

In this superfamily the bio-activity resides in the carboxy terminal half of the molecule. There is strong conservation in this region between all superfamily members, especially in 7 of the cysteine residues. The full alignment data unequivocally demonstrates that pCL13 belongs to the TGF-β superfamily. In this superfamily within family identity is of the order of 70-80%. pCL13 does not display identity of this degree to any of the individual families and therefore appears to represent an entirely new and separate category within the TGF-β superfamily.

The full nucleotide sequence and putative amino acid sequence for clone 13 (CL13) is provided at Figure 1.

EXAMPLE 2: CL13 Gene Expression and Analysis of Biological Activity

Extensive studies of regulation of CL13 gene expression have been undertaken using the ^{32}P labelled clone insert and the results are summarised at Table 1. Some examples are also illustrated in Figures 2, 3 and 4. The results indicate that the 1.2 kb transcript was present at very low levels in untreated U937 and culture derived macrophages. Expression increased markedly with phorbol 12-myristate 13-acetate (PMA), but was not upregulated by LPS or interferon-g (IFN-g). Clone 13 was expressed strongly in macrophages treated with GM-CSF, M-CSF, IL2 or TNF-a and to a lesser extent with TGF-β, PDGF-BB or IL-6. There was also increased expression of CL13 mRNA in a human neonatal fibroblast cell line (CCD34Lu) in response to IGF-1, PDGF BB, TGF-β or

TNF-α and in human umbilical vein endothelial cells grown with ECGF. No expression of this gene was found in either resting or activated B or T lymphocytes/cell lines.

We can deduce reasonable hypotheses about the nature of the biological role of this protein on the basis of its expression and the general characteristics of the superfamily. CL13 expression could be induced in culture derived macrophages (MAC) by a variety of activation agents including cytokines and PMA but not LPS. Its expression was also induced in fibroblasts by activation and could not be induced at all in lymphocytes. As the endothelial cells tested were grown in the presence of ECGS, it is not possible to conclude whether expression is absent under resting conditions.

It may be of particular significance that TGF-β induces expression of CL13 in both fibroblasts and MAC. It is possible that some of the functions ascribed to TGF-β may be due to an autocrine or paracrine induction of TGF-β by CL13.

Many of the proteins in this TGF-β superfamily act on mesenchymal cells and it is anticipated that this will be true for pCL13. It is also thought that pCL13 may enhance the effector function of these cells, perhaps in a manner similar to TGF-β itself.

Lymphocytes and macrophages are intimately related in biological function. The fact that lymphocytes do not appear able to express CL13, but MAC express it in large amounts suggests the possibility that the lymphocyte may also represent a target for pCL13 .

In summary, pCL13's properties and pattern of expression suggest that there may be some similarities to TGF-β. However, whilst it belongs to this superfamily, it can be said with some certainty, on the basis of sequence comparison, that pCL13 is one of a new class of proteins within this superfamily and is not an undescribed TGF-β protein (e.g. TGF-β6).

TABLE 1

SUMMARY OF NORTHERN BLOT ANALYSIS OF CLONE 13#

	TREATMENT	1.2 kb mRNA
Monocytoid cell lines: HL60, KG1	untreated	-
	RA or PMA	-
	RA/PMA	+
Monocytoid cell line: U937	untreated	+
	IFNg or LPS or both	+
	RA alone or with LPS	++
	PMA	+++
	RA/PMA (3 h)	++++
	RA /PMA (12 h)	+++++
	TGFb	++
	PMA/IL4	++
Macrophages [1]	untreated	-
(peripheral blood derived)	RA	+
	PMA	+++
	RA /PMA	++++
	LPS or IFN-g	-
	IFN-g followed by IL2	+
	GM CSF	+
	IL 6 or IL2 or PDGF BB or TGF b	++
	M CSF or IL1 b or TNFa	+++
B cell lines[2], T cell lines, peripheral blood T cells	with or without PMA	-
Fibroblasts (CCD 34 Lu)	nil	-
	cytokines [3]	+
Replicating endothelial cells[4]	with or without cytokines[5]	+

#Standardisation of the blots was achieved by probing with an oligonucleotide for 28S rRNA; All cell lines are human : 1. Macrophages are serum-free. 2. B cell lines were Sultan, Daudi, RPMI and U266. 3. Cytokines were IGF1, PDGF BB, TGFb and TNFa for 3 hrs. 4. HUVEC was grown with 20% FCS & growth factor (ECGF). 5. Cytokines were IFNg, TNFa, IL1b and IL2 for 3h.

EXAMPLE 3: Expression of Recombinant pCL13 and Antibody Generation

1. Prokaryotic expression of CL13

This has been undertaken using the pGEX vector which generates a glutathione-S-transferase fusion protein. Material of the correct molecular weight was synthesised but was denatured and insoluble and hence unsuitable for purification. As a consequence, no further work was done with this vector because of the difficulties that are likely to be involved.

2. Eukaryotic expression of CL13

General Approach

A number of DNA constructs based on the CL13 have been made. To some of these constructs the DNA sequence for the FLAG epitope has been added. This epitope codes for the 8 amino acid peptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) which codes for an enterokinase cleavage site is recognised by two commercially available monoclonal antibodies. A protein containing this marker peptide can then be affinity purified using these antibodies. Additionally the protein can also be detected using Western blotting or other antibody based assays. Addition of this small hydrophilic peptide of the amino terminal region of the construct would not be expected to influence the bioactivity of the whole protein. However, if desired, enterokinase can be used to selectively cleave the FLAG peptide from the construction, without affecting the rest of the molecule.

Prediction of the signal sequence cleavage site of any protein is only 75-80% accurate. For this reason in some constructions it was necessary to use the follicle stimulating hormone (FSH) leader sequence. It is known to function in the eukaryotic cell to be used for transfection and its precise cleavage site is known. This was important to ensure that the FLAG peptide remained attached to the propeptide and was not removed with signal sequence cleavage.

The following DNA constructs were made:

1. CL13: Unmodified full C13 sequence (Figure 1).
   (CL13 leader sequence-Sequence for CL13 propeptide-Sequence for mature bioactive CL13 peptide).
2. C13LB: Full length CL13 with FLAG (Figure 6).

(FSH Leader sequence-FLAG-CL-13 propeptide-Sequence for mature bioactive CL13 peptide).

3. FFC13S: Bioactive CL13 with FLAG (Figure 7)
(FSH Leader sequence-FLAG sequence-about 40 amino acids propeptide-Sequence for mature bioactive CL13 peptide).

4. C13SA: Bioactive CL13 with FLAG (Figure 8)
(FSH leader - FLAG sequence - PKA - Mature bioactive CL13). PKA is the recognition sequence for protein kinase A to allow *in vitro* phosphorylation.

These constructs were cloned into two different mammalian cell expression vectors. These are the pCEP4 vector which is a semipermanent expression vector or the pCEP4 vector from which the EBNA gene sequence has been deleted to allow it to permanently integration into the genome of the mammalian cell into which it is transfected. This allows for the development of a permanent cell line secreting this protein. The constructions have all been transferred into CHO and COS cells and either semipermanent or permanent cell lines bearing the transfectant established with the use of hygromycin to kill non transfectant bearing cells. Protein production and purification have been undertaken to date only in construction numbers 2, 3 and 4 (dominantly 3 and 4), bioactive CL13 with FLAG.

Cell Culture

Both COS and CHO cells are grown in Ham's F12 medium with 5% foetal calf serum (FCS) and 400ug/ml hygromycin (only in semipermanent cell lines). At confluence, medium is removed and replaced with HamF12 containing no serum or other supplements. The conditioned medium is removed after 3 days and used for purification of recombinant FLAG-CL13. The cells are then passaged and once more placed in serum containing medium.

Quantification

A dot blot assay has been established for quantification of recombinant FLAG containing proteins - either in culture supernatant or in purified form. Protein from culture supernatants (10-100ul) is deposited onto nitrocellulose using a dot-blot apparatus. The membrane is then

reacted with monoclonal anti-FLAG antibody and then biotinylated rabbit anti mouse IgG. This is then visualised by enhanced chemilumininescence on autoradiographic film. A standard curve is generated using a protein bacterial alkaline phosphatase (BAP) that has been engineered so that it contains 1 copy of the FLAG epitope at its amino terminus (Mr 50-55 kDa). The sensitivity of this assay is about 20ng of BAP.

When this assay was used to analyse the production of FLAG-CL13 it was found that cultures produced between about 25 and 400ng of recombinant protein per ml of culture supernatant. The best expression is seen with constructs 3 and 4.

Purification

Recombinant protein containing medium is incubated with sepharose beads to which anti-FLAG antibody has been conjugated. Approximately 1 ml of beads is used per 100 ml of conditioned medium. The sepharose and medium are incubated for 18hrs at 3deg C then beads are pelleted and poured into a minicolumn. They are then washed extensively with PBS and the recombinant protein is released with FLAG peptide. This is a very gentle but efficient procedure and ensures that the bioactivity of the recombinant protein is not damaged. The FLAG peptide is removed using gel filtration chromatography. The beads are then stripped with pH 3.5 glycine buffer and can then be re-used.

Figure 9 shows a Western blot of purified pCL13 protein from C13LB and C13SA constructs. The purified material was electrophoresed using SDS PAGE on a 15% gel under reducing and non reducing conditions prior to Western blotting and visualisation using monoclonal anti-FLAG antibody. The constructs migrate at molecular weights slightly higher than predicted, something that seems to be a function of the amino acids in the FLAG sequence and has been previously reported with the use of this epitope tag. However, there is the expected change in molecular weight associated with the use of reducing conditions indicating that the material is in the dimeric conformation.

The fact that these dimeric proteins are secreted into the medium also indicates that they are folded correctly as improperly folded and aggregated proteins expressed in eukaryotic cells are not secreted. The two constructs (FFC13SC and C13SC) which encode the bioactive protein alone,

both appear to be expressed at much higher levels than the native CL13 sequence which has only been modified to contain a FLAG epitope (C13LB). This is exemplified in Figure 9 which compares relative protein expression from constructs C13SA and C13LB.

EXAMPLE 4: Effect of pCL13 on Fibroblast Function

TGF-β stimulates fibroblast differentiated function and inhibits replication. In order to compare the function of pCL13 with TGF-β, the effect of pCL13 on fibroblast functions may be examined as follows.

a. Collagen and Glycosaminoglycan production

Neonatal lung fibroblasts (CCD34LU) can be grown to confluence and the growth medium replaced with DMEM containing 0.1% BSA. The cells can then be stimulated with recombinant pCL13 or TGF-β (10ng/ml) as a positive control. The culture supernatants can then be collected 18 hours later and assayed for total collagen and glycosaminoglycans (GAG). Collagen synthesis can be measured using a microtitre plate colorimetric assay developed in this laboratory which depends on the binding of total collagen to the dye sirius red (18). Total sulphated GAG can be measured with a colorimetric assay adapted in this laboratory for microtitre plate format and which has already been used for the *in vitro* determination of fibroblasts GAG synthesis (9,10). This assay is based on the metachromatic shift in absorption maximum for the cationic dye dimethyl-methylene blue consequent on binding the polyionic moieties of GAG (9,10).

b. Fibroblast replication

TGF-β is known to have a variable effect on in vitro fibroblast proliferation that probably depends on the balance between its capacity to down-regulate the PDGF receptor and the its induction of fibroblast PDGF synthesis. To determine whether pCL13 also modifies replication, a growth factor assay will be undertaken with CCD34Lu essentially as previously described (11, 12, 13)). These cells are sparsely plated at a concentration of about 1000 cells/ well (96 well plate). pCL13 protein or TGF-β (100ng/ml) (positive control) will be either added alone or in combination with a known

fibroblast growth factor present within fetal calf serum. Growth factor activity can be determined by ^{3}H-thymidine incorporation.

c. Collagenase activity

It would be expected that pCL13 inhibits the induction of collagenase activity. To test this, neonatal lung fibroblasts (CCD34LU) can be grown to confluence then the growth medium replaced with DMEM containing 0.1% BSA. The cells can then be stimulated with recombinant PMA to induce synthesis of collagenase in either the presence or absence of pCL13 or TGF-β (50ng/ml - positive control). The supernatants can then be assayed for collagenase activity using our adaptation (14) of an assay (15) that is based on the degradation of 20ug of purified type I collagen that has been coated onto a microtitre plate. The undigested collagen is visualized by staining with sirius red and quantified photometrically.

EXAMPLE 5: Effect of pCL13 on Macrophage Function

The effects of TGF-b on macrophages are complex and in some instances apparently paradoxical. In general terms TGF-b has been considered to be a potent macrophage chemotactic agent, a down-regulator of macrophage activation and a promoter of differentiation (3,4). To test the effect of pCL13 on macrophages, culture derived macrophages (MAC) will be used as the major cell source and will be grown free of serum in Iscove's Modified Dulbecco's Medium, using methods established by our laboratory (15,16). As replicating cells become non adherent, it is possible to utilise both adherent, and undamaged non-adherent MAC for study.

a. Chemotaxis

This may be examined using a standard Boyden chamber chemotaxis assay as previously performed (17). TGF-β (1pg/ml) will be used as the positive control for chemotaxis, and its response will be compared with that of pCL13.

b. Monocytoid cell differentiation

Both PMA and retinoic acid (RA) are potent inducers of CL13 mRNA. Both PMA, RA (as well as TGF-β) are known to induce the *in vitro*

differentiation of the primitive human monocytoid cell lines U937 and HL60 as well as bone marrow monocyte precursors. To examine the role of pCL13 in this process, the U937 and HL60 cell lines can be grown in the presence of TGF-β, pCL13 (with or without additional RA). Their differentiation will be monitored by morphology, increased adherence and inhibition of replication (^{3}H-thymidine incorporation).

It has been previously demonstrated that human MAC grow in serum free medium, and their differentiation from monocytes to macrophages in vitro can be monitored by the expression of surface CD71, the transferrin receptor (13,15). This is not seen on the surface of monocytes but is found on most MAC by day 7 of culture. Cells will be grown with TGF-b or pCL13 or interferon gamma then stained with fluoresceinated CD71 antibody and examined flow cytometrically on day 3 of culture (13). Promotion of differentiation will be associated with earlier expression of this surface antigen.

c. *Cytokine production*

TGF-β has been reported to inhibit LPS induced production of TNF-α and IL-1. Further, as TGF-β induces pCL13 expression in a number of situations, it is possible that some of the functions ascribed to TGF-β may be contributed to by pCL13. This can be examined using the above bioassays in which both TGF-β and pCL13 are active. The fibroblasts will be stimulated by TGF-β in the presence of blocking pCL13 antibody and pCL13 in the presence of a blocking TGF-β antibody. If autocrine pathways are in operation, the function in question should be reduced or inhibited by the blocking antibody. Antisense oligonucleotide inhibition experiments can also be undertaken.

EXAMPLE 6: Effect of pCL13 on Endothelial Cells

Like TGF-β, pCL13 may modify endothelial expression of adhesion molecules with subsequent downregulation of adhesion of neutrophils, monocytes or lymphocytes. Additionally pCL13 may modify angiogenesis and endothelial mediator production. This may be investigated by investigating the effect of pCL13 on:

(i) Leukocyte adherence to resting and cytokine activated vascular endothelium;

(ii) Endothelial production of cytokines such as IL-8, MCP-1, IL-1, IL-6, and endothelin;

(iii) Endothelial prostanoid synthesis;

(iv) Endothelial procoagulant activity; and

(v) Angiogenesis (in vitro and vivo).

EXAMPLE 7: Effect of pCL13 on Lymphocyte Function

Like TGF-β, pCL13 may act as an immunosuppressive agent. This can be investigated by determining the effect of pCL13 on:

(i) T and B cell proliferation;

(ii) Immunoglobulin synthesis;

(iii) LAK cell and NK cell activity; and

(iv) Production in vitro of cytokines (protein and/or mRNA) such as IL-2, IFN-g, IL-4, IL-5, IL-10.

EXAMPLE 8: Effect of pCL13 on Tumor Cell Proliferation

pCL13 may like TGF-β inhibit tumor cell replication and promote tumor differentiation. This can be investigated by determining the effect of pCL13 on:

(i) In vitro investigation of the proliferation of a wide range of tumour cell lines available through the ATCC; and

(ii) Observing change in tumor phenotype towards a more differentiated form (e.g. change from non-adherent to adherent phenotype).

EXAMPLE 9: Effect of pCL13 on Glycosaminoglycan Production by Non-transfected and transfected CHO cells.

The effect of pCL13 on glycosaminoglycan production was investigated using non-transfected and transfected CHO cells. Figure 10 shows glycosaminoglycan analysis in the non-transfected (KI) and CL13 transfected (P4N, 15 and 24) CHO cells using dimethyl-methylene blue (DMB) assay (9, 10). P4N, 15 and 24 produce increasing amounts of pCL13

respectively. Cells were cultured in DMEM/F-12 medium containing 5% FCS for 5 days. Cells were then changed to FCS-free DMEM for 24 hours. To 100 µl cell culture medium 100 µl DMB dye was added and the absorbance was read at 492 nm immediately. The results represent the mean +/- SD of triplicate wells.

EXAMPLE 10: Effect of pCL13 on Collagen Production by Non-transfected and Transfected CHO Cells

The effect of pCL13 on collagen production was investigated. Figure 11 shows the effect of pCL13 on collagen production by non-transfected (K1)and CL13 transfected (P4N, 15 and 24) CHO cells. P4N, 15 and 24 produce increasing amounts of pCL13 respectively. Cells were cultured in DMEM/F-12 medium containing 5% FCS for 5 days. Cells were then changed to FCS-free DMEM for 24 hours. The amount of collagen produced by these cells was determined using a Biodot apparatus. Culture supernatant (50 µl) was placed on nitrocellulose membrane. The membrane was washed in 100 µl PBS and dried. Collagen retained in the nitrocellulose membrane was stained with 0.1% Sirius red dye (18). Individual spots were cut out and eluted with 0.1 *N* NaOH and absorbance was read at 550 nm. The results represent the mean +/- SD of triplicate wells.

Examples 11 to 16 described hereinafter were conducted with the C13SA construct or pCL13 produced from the C13SA construct. As described above, the C13SA construct varies from CL13 in that it includes no propeptide encoding sequences.

EXAMPLE 11: Effects of pCL13 on matrix protein production

a. *Glycosaminoglycan production*

Figure 12 shows the effect of pCL13 on 35S-labelled-proteoglycan production in 3T3 (mouse fibroblasts) and neonatal human lung fibroblasts (CCD 34 Lu) after 24 hour incubation. Confluent cells were changed to RPMI culture medium containing 0.1% BSA and 50µg/ml ascorbic acid for 24 hours. Cells were then incubated with different pCL13 concentrations in the presence of 10µCi/ml [^{35}S] sulphate for 24 hours. At the end of the

incubation period medium was removed and protease inhibitors were added. Proteoglycans present in the extracellular matrix were extracted using 4M guanidine hydrochloride containing protease inhibitors for one hour at 4°C. Total proteoglycan production in the medium and the cell fraction was determined using Sephadex G-25 chromatography columns (19). The results represent the mean +/-SD of triplicate wells.

In 3T3 cells, after 24 hour incubation period, pCL13 caused a dose dependent increase in the proteoglycan production. A 92% increase was observed at 25ng/ml pCL13 concentrations and 60% increase was seen at 6.7 and 2.2 ng/ml pCL13 concentration. In comparison TGF-β at 20ng/ml elevated proteoglycan production by 95%. In CCD 34Lu cells, pCL13 at 50ng/ml caused 23% increase in the proteoglycan production and 6% increase at 25ng/ml pCL13 concentration. In comparison TGF-β at 10ng/ml elevated proteoglycan production by 36%.

b. *Collagen production*

Figure 13 shows the effect of pCL13 on collagen type 1 production in neonatal lung fibroblasts (CCD 34 Lu). Confluent cells were changed to DMEM culture medium containing 0.1% BSA and 50μg/ml ascorbic acid for 24 hours. Cells were then incubated with different pCL13 concentrations in the presence of 50μg/ml b-aminopropionitrile for 24 hours. At the end of incubation period the amount of collagen present in the medium was determined using an ELISA. Briefly, supernatants from treated and non-treated fibroblasts as well as type 1 collagen standards were incubated for 72 hours at 4°C in 96-well microtitre plates (NUNC). At the end of incubation period plates were washed, blocked with 4% bovine serum albumin in phosphate buffered saline, incubated with collagen type 1 monoclonal antibody (Sigma). The plates were then rewashed and biotinylated mouse IgG was added and followed by streptavidin complex. After the addition of substrate, plates were read at 490/405 nm on a plate reader. The results represent the mean +/-SD of triplicate wells. pCL13 at 50ng/ml caused 140% increase in the collagen production, 190% increase at 25ng/ml and 11% at 5ng/ml concentration. In comparison TGF-β at 10ng/ml elevated collagen production by 34% after 24 hours. The relatively poor TGF-β response has occurred because TGF-β requires 48-78 hours to achieve maximal effect.

EXAMPLE 12: Effect of pCL13 on fibroblast replication

a. Growth under limiting serum conditions

The growth factor activity of pCL13 and transforming growth factor beta (TGFβ) on growth-arrested BFF (human baby foreskin fibroblasts) and 3T3 (mouse fibroblasts) cells was determined. The cytokines were added to BFF and 3T3 cells in 0.2% foetal bovine serum (FBS) media to determine whether they were true growth factors which could stimulate a resting cell to progress through the cell cycle and undergo division. The growth factor assay was performed as previously described (11, 12). In brief, the cells were plated at $1.2x10^3$ cells/well in 200mL of growth-arresting medium (0.2% FBS) for 72h. The media was then replaced with fresh 0.2% FBS media with or without cytokines and 0.5mCi/well of [3-H] Thymidine for a further 72h. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells was counted on a liquid scintillation analyser. The controls included 0.2% FBS media only, 10% FBS media only (normal growth media), and pCL13 diluent (0.1% CHAPS) in 0.2% FBS media.

The results shown in Figure 14 indicate that pCL13 appears to have true growth factor activity on both human BFF. TGFβ appears to be inhibitory for BFF cells. Neither pCL13 not TGFβ exhibit growth factor activity on 3T3 under the conditions of this assay.

b. Growth in the presence of serum

The growth factor activity of pCL13 and transforming growth factor beta (TGFβ) on growth-arrested BFF (human baby foreskin fibroblasts) and 3T3 (mouse fibroblasts) cells was determined. The cytokines were added to BFF and 3T3 cells in 2% foetal bovine serum (FBS) media to determine whether they were growth enhancing substances which could enhance the rate at which the cells moved through the cell cycle. The growth factor assay was performed as previously described (11, 12). In brief, the cells were plated at $1.2x10^3$ cells/well in 200ml of growth-arresting medium (0.2% FBS) for 72h. The media was then replaced with fresh 2% FBS media with or without cytokines and 0.5mCi/well of [3-H] Thymidine for a further 72h. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells was counted on a liquid

scintillation analyser. The controls included 2% FBS media only, 10% FBS media only (normal growth media), and pCL13 diluent (0.1% CHAPS) in 0.2% FBS media.

The results (Figure 15) show that pCL13 had a growth-enhancing effect on human BFF and murine 3T3 cells. TGFβ appears to be inhibitory for BFF cells but to have growth-enhancing activity at low concentration on 3T3 cells.

EXAMPLE 13: Effects of pCL13 on replication of human monocytoid cell lines

pCL13 was compared with transforming growth factor beta (TGFb) and interferon alpha 2b (IFNa2b), for their antiproliferative effect on the cell line U937 (a human monocyte-like histiocytic lymphoma) and Mono Mac 6 (a monoblastic leukemia cell line). The cells were plated at $3x10^4$ cells/well in 200mL of 10% FBS (foetal bovine serum) medium with or without cytokines. For the final 6h of a 48h incubation period, the wells were pulsed with 0.5mCi/well of [3-h] Thymidine. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells were counted on a liquid scintillation analyser. The controls include 10% FBS medium alone and pCL13 diluent in 10% FBS medium.

The results (Figure 16) indicate that two batches of pCL13, B447 and B448A, at concentrations of 10 and 100ng/ml have a small antiproliferative effect on two human cell lines of monocytic origin. This contrasts with the stronger antiproliferative effects of TGFβ (2 and 20ng/ml) and IFNa2b (10^3 and 10^5 U/ml) on U937 and Mono Mac 6 cells.

EXAMPLE 14: Effects of pCL13 on macrophage production of TNF

The data in Figure 17 shows the effect of different pCL13 concentration on LPS stimulated TNF-α production from human culture derived macrophages. Monocytes were purified by elutriation from buffy coats and cultured in Iscove's medium containing 0.1% BSA (13, 16). On day 5, cells were incubated with different pCL13 concentrations in the presence of 10μg/ml LPS in the Iscove's medium for 24 hours. At the end of incubation period medium was removed and the amount of TNF-α present

was determined using a sandwich ELISA (Genzyme). The results show that pCL13 caused inhibition of LPS induced TNF-α production. A 47% inhibition was observed at 20ng/ml pCL13 and a 27% inhibition was seen at 7ng/ml of pCL13. In comparison TGF-β only brought about 10% reduction at 20ng/ml.

EXAMPLE 15: Effects of pCL13 on tumor cytotoxicity

The direct effect of pCL13 and TGFβ on tumour target cells (5637 bladder carcinoma and MDA-MB-231 breast adenocarcinoma) and the effect of pCL13 and TGFβ on monocyte-mediated killing of tumor cells was examined by measuring the release of radiolabelled DNA from lysed tumour target cells. The cytotoxicity assay was performed as previously described (20). Tumour target cells (labelled while in the exponential growth phase with 20μCi of [^{3}H] Thymidine/1x10^6 cells for 24h) were added to the monocytes (effectors) at an effector:target (E:T) ratios of 10:1 for 72 h. The cells were then centrifuged and the supernatants counted in scintillation fluid on a liquid scintillation analyser. The controls included untreated tumour cells, untreated tumour cells co-cultured with monocytes and cytokine diluent alone. TGFβ and pCL13 were incubated with monocytes for 48h.

The results are shown at Figure 18. Neither pCL13 nor TGFβ had a direct cytotoxic effect on the 5637 or MDA-MB-231 tumour lines. However pCL13 enhanced the ability of monocytes to kill 5637 cells. pCL13 also enhanced the monocyte-mediated killing of T24 (bladder carcinoma), J82 (bladder carcinoma), T47D (breast ductal carcinoma) and JCPL (ovarian carcinoma)(data not shown).

EXAMPLE 16: *In vivo* Effects of pCL13

Rats (Fisher F343) were injected subcutaneously on their backs with 0.1ml of three concentrations of pCL13, a negative saline control and TGFβ. The injections were widely separated and each animal was administered with the whole panel of 5 injections. The amounts of pCL13 injected were 60ng, 30ng and 2ng. The dose of TGFβ administered was 10ng. The animals were then sacrificed at intervals commencing at 3 hours and up to 2 weeks

following administration. Three animals were used for each time point, and following sacrifice the areas in which material had been administered was excised, formalin fixed, mounted, then stained with haematoxylin and eosin. The material was then evaluated microscopically.

a. *Macroscopic Changes*

There was no macroscopic difference between the biopsies in any of the animals, under any of the various conditions other than at the two week time point. At the two week time point however, the biopsies, only of the areas with the two highest doses of pCL13, showed obvious macroscopic differences in the area between the muscle and skin. This area seemed somewhat expanded and had a white glistening appearance, suggestive of excess matrix protein deposition.

b. *Microscopic Evaluation*

No differences were seen on histological sections at the three hour time points. However at the day one (24 hour) time point the areas in which the two highest concentrations of pCL13 had been administered demonstrated a mononuclear cell infiltrate which was somewhat patchy in character and was present dominantly in the subcutaneous tissue (Figure 19A). No similar changes were observed in either the negative saline control (Figure 19B) or TGFβ at a dose of 10ng/ml. The infiltrate seemed to be present maximally at days one and two and be markedly diminished or absent from day four onwards. These findings suggest that pCL13 was chemotactic for macrophages and or lymphocytes.

This study was not undertaken in such a manner as to be able to supply good quantitative data on the amount of collagen that was present in the areas where the two substances were administered. However in conjunction with the macroscopic appearance, it appears likely that the amount of collagen was increased in the samples containing the two highest doses of clone 13, at least at the two week time point.

EXAMPLE 17: Clone 13 Variants

Re-screening was undertaken using a fetal lung cDNA library using a portion of the coding sequence of clone 13 as a probe. This was undertaken

in order to determine the existence of clone 13 variants. Using this approach a number of additional clones (a1, b2, h1, b1, d2, dd2, f1 and u2) were obtained and the sequence of these clones is illustrated in Figure 20A which shows the nucleotide sequence and Figure 20B which shows a portion of the translated open reading frame. It also compares the sequences with that of the original clone 13 sequence (C13). From Figure 20B, it can be seen that the translated coding region of these clone 13 variants displays only minor differences. These occur at amino acids 9, 48 and 202. These are all in the propeptide region and are likely to represent genetic differences between the individuals whose RNA was used to prepare the cDNA library. However, at the DNA level, there is substantial variation dominantly in the 5' untranslated region, but to a lesser extent in the 3' untranslated region. Whilst these variants may well be important in areas such as transcriptional regulation they are untranslated and hence cannot affect bioactivity.

Some of the clones isolated and displayed in Figure 20A (e.g. b2 and h1) even though they have very long 5' untranslated region, still do not represent the complete coding sequence. This can be ascertained as when the 5' untranslated region is used as a probe, on northern blots, hybridisation to a band of approximately 7kb is demonstrable. The reasons for this marked length variation are unclear but could include alternate splicing of an untranslated exon, the use of alternate transcriptional start sites or even gene duplication.

EXAMPLE 18: Expression of clone is using a yeast eukaryotic system

The bioactive region of clone 13, modified at its amino terminus so as to contain a number of additional marker epitopes (construct C13SA/5H - Figure 21) was cloned into the pPIC9 plasmid. This plasmid was then used to transform the yeast *Pichia pastoris* according to the manufacturers instructions (Invitrogen Corp.). Yeast, successfully transformed by this plasmid were selected on the basis of methanol sensitivity. Colonies of yeast were then grown for two days, in suspension as per the manufacturers instructions. Culture medium was collected and an aliquot subjected to SDS-PAGE followed by western blotting. pCL13 containing bands were visualised using the anti-FLAG M2 antibody using standard procedures. Electrophoresis was carried out under both reducing and non-reducing

conditions. It can be seen that large amounts of the protein are produced which are easily detectable with unconcentrated yeast culture medium, indicating secretion of the protein in an appropriate manner (Figure 22). The molecular weight approximates that expected on the basis of the amino acid composition and the doubling of the molecular weight under non-reducing conditions (Figure 22) indicates that the protein is, as expected, a disulphide bonded dimer. This is the correct structural configuration and indicates that the protein has been processed and secreted by the yeast organism in an appropriate manner.

The capacity to express this complex dimeric, protein with a high disulphide bond content in yeast systems is highly advantageous as it dramatically lowers the cost of production per unit quantity of protein and makes it far more suitable as a biopharmaceutical compared with material produced by mammalian cells.

Whilst this work has been undertaken with the yeast *Pichia pastoris*, it is quite likely that similar secretion will occur with a range of yeast organisms transduced with an appropriate yeast expression vector. As the bioactive region being expressed does not contain potential n-glycosylation sites, the hyperglycosylation, that sometimes occurs with mammalian proteins expressed by yeast strains, is not an issue.

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It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:-

1. An isolated polynucleotide molecule comprising a nucleotide sequence encoding, or complementary to a nucleotide sequence encoding, a protein designated pCL13 or a biologically active fragment thereof.

2. An isolated polynucleotide molecule comprising a nucleotide sequence substantially corresponding to that shown in Figure 1, a portion thereof which encodes a biologically active fragment of pCL13, or a nucleotide sequence complementary thereto.

3. An isolated polynucleotide molecule comprising a nucleotide sequence substantially corresponding to a mutant, variant or derivative sequence of that shown in Figure 1, or a nucleotide sequence complementary thereto.

4. A polynucleotide molecule according to claim 3, wherein the nucleotide sequence substantially corresponds to a variant nucleotide sequence selected from a1, b1, b2, d2, dd2, f1, h1 and u2 as shown in Figure 20A, or a nucleotide sequence complementary thereto.

5. A polynucleotide molecule according to claim 1, wherein the protein comprises a polypeptide having an amino acid sequence selected from those shown in Figure 20B.

6. A polynucleotide molecule according to claim 1, wherein the protein comprises a polypeptide having an amino acid sequence substantially as shown in Figure 1.

7. An isolated polynucleotide molecule comprising a nucleotide sequence hybridisable to the nucleotide sequence shown in Figure 1 under medium stringency conditions.

8. An isolated polynucleotide molecule comprising a nucleotide sequence hybridisable to the nucleotide sequence shown in Figure 1 under high stringency conditions.

9. A polynucleotide molecule according to claim 7 or 8 capable of being utilised as a probe or primer for a polynucleotide sequence encoding a protein designated pCL13.

10. A polynucleotide molecule according to claim 7 or 8 being of a length greater than 10 nucleotides.

11. An isolated polynucleotide molecule comprising a nucleotide sequence having at least 70% homology to the nucleotide sequence shown in Figure 1.

12. An isolated polynucleotide molecule comprising a nucleotide sequence having at least 90% homology to the nucleotide sequence shown in Figure 1.

13. A polynucleotide molecule according to any one of the preceding claims, wherein the nucleotide sequence encoding the pCL13 or biologically active fragment thereof does not comprise sequence encoding the pCL13 leader or propeptide.

14. A polynucleotide molecule according to claim 13, wherein said nucleotide sequence encoding the pCL13 or biologically active fragment thereof includes sequence encoding a heterologous leader.

15. A polynucleotide molecule according to claim 14, wherein said heterologous leader is the follicle stimulating hormone (FSH) leader.

16. A polynucleotide molecule according to any one of the preceding claims, wherein the nucleotide sequence encoding the pCL13 or biologically active fragment thereof includes sequence encoding an epitope tag.

17. A polynucleotide molecule according to claim 16, wherein the epitope tag is FLAG and/or HIS.

18. A polynucleotide molecule according to any one of the preceding claims, wherein the polynucleotide molecule is DNA.

19. A vector comprising a DNA molecule according to claim 18 operably linked to a suitable promoter.

20. A vector comprising a DNA molecule according to claim 19, the DNA molecule being operably linked in opposite orientation to a suitable promoter such that expression proceeds 5' to the 3' terminus to produce antisense RNA.

21. A vector according to claim 20, wherein said DNA molecule includes or is linked to a nucleotide sequence encoding a ribozyme domain.

22. A protein designated pCL13 in substantially pure form.

23. A protein according to claim 22, wherein the protein comprises a monomeric polypeptide(s) having an amino acid sequence selected from those shown in Figure 20B.

24. A protein according to claim 22, wherein the protein comprises a monomeric polypeptide(s) having an amino acid sequence substantially as shown in Figure 1.

25. A biologically active fragment of a protein according to any one of claims 22 to 24.

26. A biologically active fragment according claim 25, wherein said biologically active fragment corresponds to a pCL13 propeptide or fragment thereof.

27. A protein or antigenic portion thereof, which binds to an anti-pCL13 antibody.

28. A non-human organism transformed with a polynucleotide molecule according to any one of claims 1 to 18 or a vector according to any one of claims 19 to 21.

29. A non-human organism according to claim 28 selected from eukaryotic cell lines, yeast, animals and plants.

30. An antibody or fragment thereof which specifically binds to the protein designated pCL13 or an antigenic portion thereof.

31. A method of producing a protein designated pCL13 or a biologically active fragment thereof, comprising transforming a suitable host organism with a polynucleotide molecule according to any one of claims 1 to 12, wherein said polynucleotide molecule is constitutively or inducibly expressed in said host organism.

32. A method of producing a protein designated pCL13 or a biologically active fragment thereof, comprising transforming a suitable host organism with a polynucleotide molecule according to any one of claims 13 to 17, wherein said polynucleotide molecule is constitutively or inducibly expressed in said host organism.

33. A method according to claim 31 or 32, wherein said host organism is selected from eukaryotic cell lines and yeast.

34. A method according to claim 33, wherein said host organism is a yeast.

35. A method according to claim 34, wherin said yeast is *Pichia pastoris*.

36. A method of treatment of a disease or condition in a subject which is beneficially treatable with TGF-β, comprising administering to said subject a preparation comprising a protein or biologically active fragment thereof according to any one of claims 22 to 27 or, alternatively, an agent for reducing the expression or activity of native pCL13, optionally in admixture with a pharmaceutically acceptable carrier.

37. A method of treatment of a disease or condition in a subject, said disease or condition being selected from wound and/or fracture healing, ischaemic injury, cancer, autoimmune diseases, chronic inflammatory diseases, imunosuppresion, fibrotic/fibroproliferative disorders such as rheumatoid arthritis, artherosclerosis, pulmonary fibrosis, scleroderma, liver cirrhosis and keloids, comprising administering to said subject a preparation comprising a protein or biologically active fragment thereof according to any one of claims 22 to 27 or, alternatively, an agent for reducing the expression or activity of native pCL13, optionally in admixture with a suitable pharmaceutically acceptable carrier.

38. A method for diagnosing a disease or condition in a subject, said disease or condition being selected from inflammatory and fibrotic diseases, comprising detecting the presence or activity of the protein designated pCL13 in said subject.

39. A kit for use in a method according to claim 38, said kit comprising a protein or biologically active fragment thereof according to any one of claims 22 to 27, or an antibody or fragment thereof according to claim 31.

40. A gene therapy agent comprising a polynucleotide molecule according to any one of claims 1 to 18 or a vector according to any one of claims 19 to 21.

41. A receptor molecule specific for a protein designated pCL13, in substantially pure form.

```
   1 GCGGCCGCTGCACAGCC ATG CCC GGG CAA GAA CTC AGG ACG CTG AAT GGC TCT CAG ATG CTC    62
   1                   M   P   G   Q   E   L   R   T   L   N   G   S   Q   M   L     15

  63 CTG GTG TTG CTG GTG CTC TCG TGG CTG CCG CAT GGG GGC GCC CTG TCT CTG GCC GAG GCG  122
  16 L   V   L   L   V   L   S   W   L   P   H   G   G   A   L   S   L   A   E   A   35

 123 AGC CGC GCA AGT TTC CCG GGA CCC TCA GAG TTG CAC ACC GAA GAC TCC AGA TTC CGA GAG  182
  36 S   R   A   S   F   P   G   P   S   E   L   H   T   E   D   S   R   F   R   E   55

 183 TTG CGG AAA CGC TAC GAG GAC CTG CTA ACC AGG CTG CGG GCC AAC CAG AGC TGG GAA GAT  242
  56 L   R   K   R   Y   E   D   L   L   T   R   L   R   A   N   Q   S   W   E   D   75

 243 TCG AAC ACC GAC CTC GTC CCG GCC CCT GCA GTC CGG ATA CTC ACG CCA GAA GTG CGG CTG  302
  76 S   N   T   D   L   V   P   A   P   A   V   R   I   L   T   P   E   V   R   L   95

 303 GGA TCC GGC GGC CAC CTG CAC CTG CGT ATC TCT CGG GCC GCC CTT CCC GAG GGG CTC CCC  362
  96 G   S   G   G   H   L   H   L   R   I   S   R   A   A   L   P   E   G   L   P  115

 363 GAG GCC TCC CGC CTT CAC CGG GCT CTG TTC CGG CTG TCC CCG ACG GCG TCA AGG TCG TGG  422
 116 E   A   S   R   L   H   R   A   L   F   R   L   S   P   T   A   S   R   S   W  135

 423 GAC GTG ACA CGA CCT CTG CGG CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG  482
 136 D   V   T   R   P   L   R   R   Q   L   S   L   A   R   P   Q   A   P   A   L  155

 483 CAC CTG CGA CTG TCG CCG CCG CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC  542
 156 H   L   R   L   S   P   P   P   S   Q   S   D   Q   L   L   A   E   S   S   S  175

 543 GCA CGG CCC CAG CTG GAG TTG CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG  602
 176 A   R   P   Q   L   E   L   H   L   R   P   Q   A   A   R   G   R   R   R   A  195
       ↓
 603 CGT GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG CGT TGC TGC CGT CTG CAC ACG  662
 196 R   A   R   N   G   D   H   C   P   L   G   P   G   R   C   C   R   L   H   T  215

 663 GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG GTG CTG TCG CCA CGG GAG GTG  722
 216 V   R   A   S   L   E   D   L   G   W   A   D   W   V   L   S   P   R   E   V  235

 723 CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC CGG GCG GCA AAC ATG CAC GCG  782
 236 Q   V   T   M   C   I   G   A   C   P   S   Q   F   R   A   A   N   M   H   A  255

 783 CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG GTG CCA GCG CCC TGC TGC GTG  842
 256 Q   I   K   T   S   L   H   R   L   K   P   D   T   V   P   A   P   C   C   V  275

 843 CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC GAC ACC GGG GTG TCG CTC CAG  902
 276 P   A   S   Y   N   P   M   V   L   I   Q   K   T   D   T   G   V   S   L   Q  295

 903 ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA TGA GCAGTCCTGGTCCTTCCACTGTGC 968
 296 T   Y   D   D   L   L   A   K   D   C   H   C   I   •                         309

 969 ACCTGCGCGGGGGAGGCGACCTCAGTTGTCCTGCCCTGTGGA ATG GGC TCA AGG TTC CTG AGA CAC CCG  1037

1038 ATT CCT GCC CAA ACA GCT GTA TTT ATA TAA GTCTGTTATTTATTATTAATTTATTGGGGTGACCTTCTTG 1107

1108 GGGACTCGGGGGCTGGTCTG ATG GAA CTG TGT ATT TAT TTA AAA CTC TGG TGA TAAAAATAAAGCTGT 1175

1176 CTGAACTGTTAAAAAAAAAAAAAAAA                                                    1202
```

↓ Processing site following nucleotide 605
• Stop codon

FIGURE 1

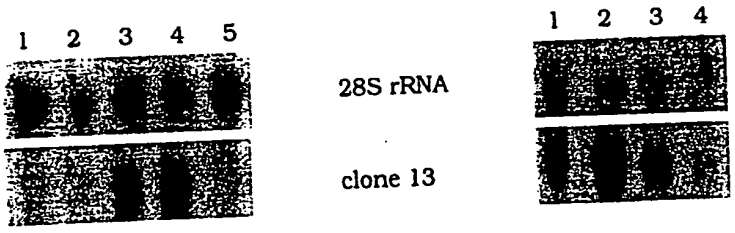


FIGURE 2

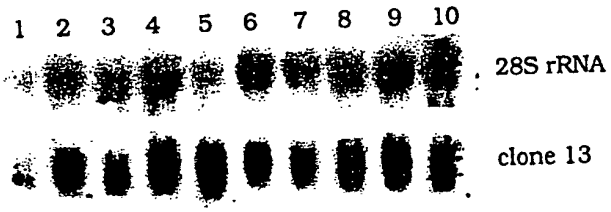


FIGURE 3

FIGURE 4

```
CBMP2_HUM       CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIVQTLVN
CBMP3_HUM       CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPKSLKPSNHATIQSIVR
CBMP4_HUM       CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAIVQTLVN
CBMP5_HUM       CKKHELYVSFR-DLGWQDWIIAPEGYAAFYCDGECSFPLNAHMNATNHAIVQTLVH
CBMP6_HUM       CRKHELYVSFQ-DLGWQDWIIAPKGYAANYCDGECSFPLNAHMNATNHAIVQTLVH
CBMP7_HUM       CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAIVQTLVH
CGDF3_MOUS      CHRHQLFINFQ-DLGWHKWVIAPKGFMANYCHGECPFSMTTYLNSSNYAFMQALMH
CGDF9_MOUS      CELHDFSLSFS-QLKWDNWIVAPHSYNPSYCKGDCPSAVSHRYGSPVHIMVQNMIY
CHSOP1_5        CKKHELYVSFR-DLGWQDWIIAPEGYA---------FPLNSYMNATNHAIVQTLVH
CIHBA_HUM       CCKKQFFVSFK-DIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSLSFHSTVINHYRM
CMIS_HUM        CALRELSVDLRAERS----VLIPETYQANNCQGVCGWPQSDRNPRYGNHVVLLLKM
CPEP13          CCRLHIVRASLEDLGWADWVLSPREVQVTMCIGACP---SQFRAANMHAQIKTSLH
CTGF1_HUM       CCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS---LDTQYSKVLALYN
CTGF2_HUM       CCLRPLYIDFKRDLGWK-WIHEPKGYNANFCAGACPYLWS---SDTQHSRVLSLYN
CTGF3_HUM       CCVRPLYIDFRQDLGWK-WVHEPKGYYANFCSGPCPYLRS---ADTTHSTVLGLYN
CTGF4_CHIC      CCVRPLYIDFRKDLGWK-WIHEPKGYMANFCMGPCPYIWS---ADTQYTKVLALYN
CTGF5_X         CCVKPLYINFRKDLGWE----------ANYCLGNCPYIWS---MDTQYSKVLSLYN
CVG1_X          CKKRHLYVEFK-DVGWQNWVIAPQGYMANYCYGECPYPLTEILNGSNHAILQTLVH
                *

CBMP2_HUM       SVNSK--IPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR
CBMP3_HUM       AVGVVPGIPEPCCVPEKMSSLSILFFDENKNVVLKVYPNMTVESCACR
CBMP4_HUM       SVNSS--IPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
CBMP5_HUM       LMFPDH-VPKPCCAPTKLNAISVLYFDDSSNVILKKYRNMVVRSCGCH
CBMP6_HUM       LMNPEY-VPKPCCAPTKLNAISVLYFDDNSNVILKKYRNMVVRACGCH
CBMP7_HUM       FINPET-VPKPCCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH
CGDF3_MOUS      MADP-K-VPKAVCVPTKLSPISMLYQDSDKNVILRHYEDMVVDECGCG
CGDF9_MOUS      E-KLDPSVPSPSCVPGKYSPLSVLTIEPDGSIAYKEYEDMVATSCTCR
CHSOP1_5        FINPET-VPKPCCAPTQLNAISV---------ILKKYRNMVVRACGCH
CIHBA_HUM       RGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS
CMIS_HUM        QARGAALARPPCCVPTAYAG-KLLISLSEERISAHHVPNMVATECGCR
CPEP13          RLKPDT-VPAPCCVPASYNPM-VLIQKTDTGVSLQTYDDLLAKDCHCI
CTGF1_HUM       QHNPGASAA-PCCVPQALEPLPIVYY-VGRKPKVEQLSNMIVRSCKCS
CTGF2_HUM       TINPEASAS-PCCVSQDLEPLTILYY-IGKTPKIEQLSNMIVKSCKCS
CTGF3_HUM       TLNPEASAS-PCCVPQDLEPLTILYY-VGRTPKVEQLSNMVVKSCKCS
CTGF4_CHIC      QHNPGASAA-PCCVPQILDPLPIIYY-VGRNVRVEQLSNMVVRACKCS
CTGF5_X         QNNPGASIS-PCCVP---------YY-VGRTAKVEQLSNMVVRSCNCS
CVG1_X          SIEPED-IPLPCCVPTKMSPISMLFYDNNDNVVLRHYENMAVDECGCR
```

FIGURE 5

```
   1 AAGCTT ATG CCC GGG CAA GAA CTC AGG ACG CTG AAT GGC TCT CAG ATG CTC CTG GTG TTG   60
   1        M  P  G  Q  E  L  R  T  L  N  G  S  Q  M  L  L  V  L                    18

  61 CTG GTG CTC TCG TGG CTG CCG CAT GGG GGC GCC CTG TCT CTG GCC GAG GCG AGC CGC GCA  120
  19 L  V  L  S  W  L  P  H  G  G  A  L  S  L  A  E  A  S  R  A                    38

 121 AGT TTC CCG GGA CCC TCA GAG TTG CAC ACC GAA GAC TCC AGA TTC CGA GAG TTG CGG AAA  180
  39 S  F  P  G  P  S  E  L  H  T  E  D  S  R  F  R  E  L  R  K                    58

 181 CGC TAC GAG GAC CTG CTA ACC AGG CTG CGG GCC AAC CAG AGC TGG GAA GAT TCG AAC ACC  240
  59 R  Y  E  D  L  L  T  R  L  R  A  N  Q  S  W  E  D  S  N  T                    78

 241 GAC CTC GTC CCG GCC CCT GCA GTC CGG ATA CTC ACG CCA GAA GTG CGG CTG GGA TCC GGC  300
  79 D  L  V  P  A  P  A  V  R  I  L  T  P  E  V  R  L  G  S  G                    98

 301 GGC CAC CTG CAC CTG CGT ATC TCT CGG GCC GCC CTT CCC GAG GGG CTC CCC GAG GCC TCC  360
  99 G  H  L  H  L  R  I  S  R  A  A  L  P  E  G  L  P  E  A  S                   118

 361 CGC CTT CAC CGG GCT CTG TTC CGG CTG TCC CCG ACG GCG TCA AGG TCG TGG GAC GTG ACA  420
 119 R  L  H  R  A  L  F  R  L  S  P  T  A  S  R  S  W  D  V  T                   138

 421 CGA CCT CTG CGG CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG CAC CTG CGA  480
 139 R  P  L  R  R  Q  L  S  L  A  R  P  Q  A  P  A  L  H  L  R                   158

 481 CTG TCG CCG CCG CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC GCA CGG CCC  540
 159 L  S  P  P  P  S  Q  S  D  Q  L  L  A  E  S  S  S  A  R  P                   178
                                                              ↓
 541 CAG CTG GAG TTG CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG CGT GAA TTC  600
 179 Q  L  E  L  H  L  R  P  Q  A  A  R  G  R  R  R  A  R  E  F                   198

 601 GAC TAC AAG GAC GAC GAT GAC AAG GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG  660
 199 D  Y  K  D  D  D  D  K  A  R  N  G  D  H  C  P  L  G  P  G                   218

 661 CGT TGC TGC CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG  720
 219 R  C  C  R  L  H  T  V  R  A  S  L  E  D  L  G  W  A  D  W                   238

 721 GTG CTG TCG CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC  780
 239 V  L  S  P  R  E  V  Q  V  T  M  C  I  G  A  C  P  S  Q  F                   258

 781 CGG GCG GCA AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG  840
 259 R  A  A  N  M  H  A  Q  I  K  T  S  L  H  R  L  K  P  D  T                   278

 841 GTG CCA GCG CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC  900
 279 V  P  A  P  C  C  V  P  A  S  Y  N  P  M  V  L  I  Q  K  T                   298

 901 GAC ACC GGG GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA  960
 299 D  T  G  V  S  L  Q  T  Y  D  D  L  L  A  K  D  C  H  C  I                   318

 961 TGA CTCGAG                                                                    969
 319 •                                                                             319
```

↓ = processing site
underline = FLAG epitope that is fused to amino terminus of bioactive region of clone 13
• = stop codon

FIGURE 6

```
   1 GCTAGCGCC ATG GAT TAC TAC AGA AAA TAT GCA GCT ATC TTT CTG GTC ACA TTG TCG GTG    60
   1          M  D  Y  Y  R  K  Y  A  A  I  F  L  V  T  L  S  V                    17
                                ⬇
  61 TTT CTG CAT GTT CTC CAT TCC GCT CCT GAT GAA TTC GAC TAC AAG GAC GAC GAT GAC AAG 120
  18 F  L  H  V  L  H  S  A  P  D  E  F  D  Y  K  D  D  D  D  K                   37

 121 CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG CAC CTG CGA CTG TCG CCG CCG 180
  38 R  Q  L  S  L  A  R  P  Q  A  P  A  L  H  L  R  L  S  P  P                   57

 181 CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC GCA CGG CCC CAG CTG GAG TTG 240
  58 P  S  Q  S  D  Q  L  L  A  E  S  S  S  A  R  P  Q  L  E  L                   77
                                                   ▼
 241 CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG CGT GCG CGC AAC GGG GAC CAC 300
  78 H  L  R  P  Q  A  A  R  G  R  R  R  A  R  A  R  N  G  D  H                   97

 301 TGT CCG CTC GGG CCC GGG CGT TGC TGC CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC 360
  98 C  P  L  G  P  G  R  C  C  R  L  H  T  V  R  A  S  L  E  D                  117

 361 CTG GGC TGG GCC GAT TGG GTG CTG TCG CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC 420
 118 L  G  W  A  D  W  V  L  S  P  R  E  V  Q  V  T  M  C  I  G                  137

 421 GCG TGC CCG AGC CAG TTC CGG GCG GCA AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC 480
 138 A  C  P  S  Q  F  R  A  A  N  M  H  A  Q  I  K  T  S  L  H                  157

 481 CGC CTG AAG CCC GAC ACG GTG CCA GCG CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG 540
 158 R  L  K  P  D  T  V  P  A  P  C  C  V  P  A  S  Y  N  P  M                  177

 541 GTG CTC ATT CAA AAG ACC GAC ACC GGG GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC 600
 178 V  L  I  Q  K  T  D  T  G  V  S  L  Q  T  Y  D  D  L  L  A                  197

 601 AAA GAC TGC CAC TGC ATA TGA CTCGAG                                           627
 198 K  D  C  H  C  I  •                                                         204
```

underline = FLAG epitope

⬇ = signal sequence cleavage site following first 24 amino acids representing the FSH leader sequence

• = stop codon

▼ = predicted processing site is NOT used and the protein secreted commences after the signal sequence at amino acid 25

FIGURE 7

```
   1 GCTAGCGCC ATG GAT TAC TAC AGA AAA TAT GCA GCT ATC TTT CTG GTC ACA TTG TCG GTG    60
   1          M  D  Y  Y  R  K  Y  A  A  I  F  L  V  T  L  S  V                    17
                                  ↓
  61 TTT CTG CAT GTT CTC CAT TCC GCT CCT GAT GAA TTC GAC TAC AAG GAC GAC GAC GAC AAG  120
  18 F  L  H  V  L  H  S  A  P  D  E  F  D  Y  K  D  D  D  D  K                    37

 121 [CTC CGC GCC TCC GTG]GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG CGT TGC TGC  180
  38 [L  R  A  S  V ]A  R  N  G  D  H  C  P  L  G  P  G  R  C  C                    57

 181 CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG GTG CTG TCG  240
  58 R  L  H  T  V  R  A  S  L  E  D  L  G  W  A  D  W  V  L  S                    77

 241 CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC CGG GCG GCA  300
  78 P  R  E  V  Q  V  T  M  C  I  G  A  C  P  S  Q  F  R  A  A                    97

 301 AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG GTG CCA GCG  360
  98 N  M  H  A  Q  I  K  T  S  L  H  R  L  K  P  D  T  V  P  A                   117

 361 CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC GAC ACC GGG  420
 118 P  C  C  V  P  A  S  Y  N  P  M  V  L  I  Q  K  T  D  T  G                   137

 421 GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA TGA CTCGAG  480
 138 V  S  L  Q  T  Y  D  D  L  L  A  K  D  C  H  C  I  •                         155
```

underline = FLAG epitope
[ ] = PKA site
↓ = signal sequence cleavage site following first 24 amino acids representing the FSH leader sequence
• = stop codon

FIGURE 8

Figure

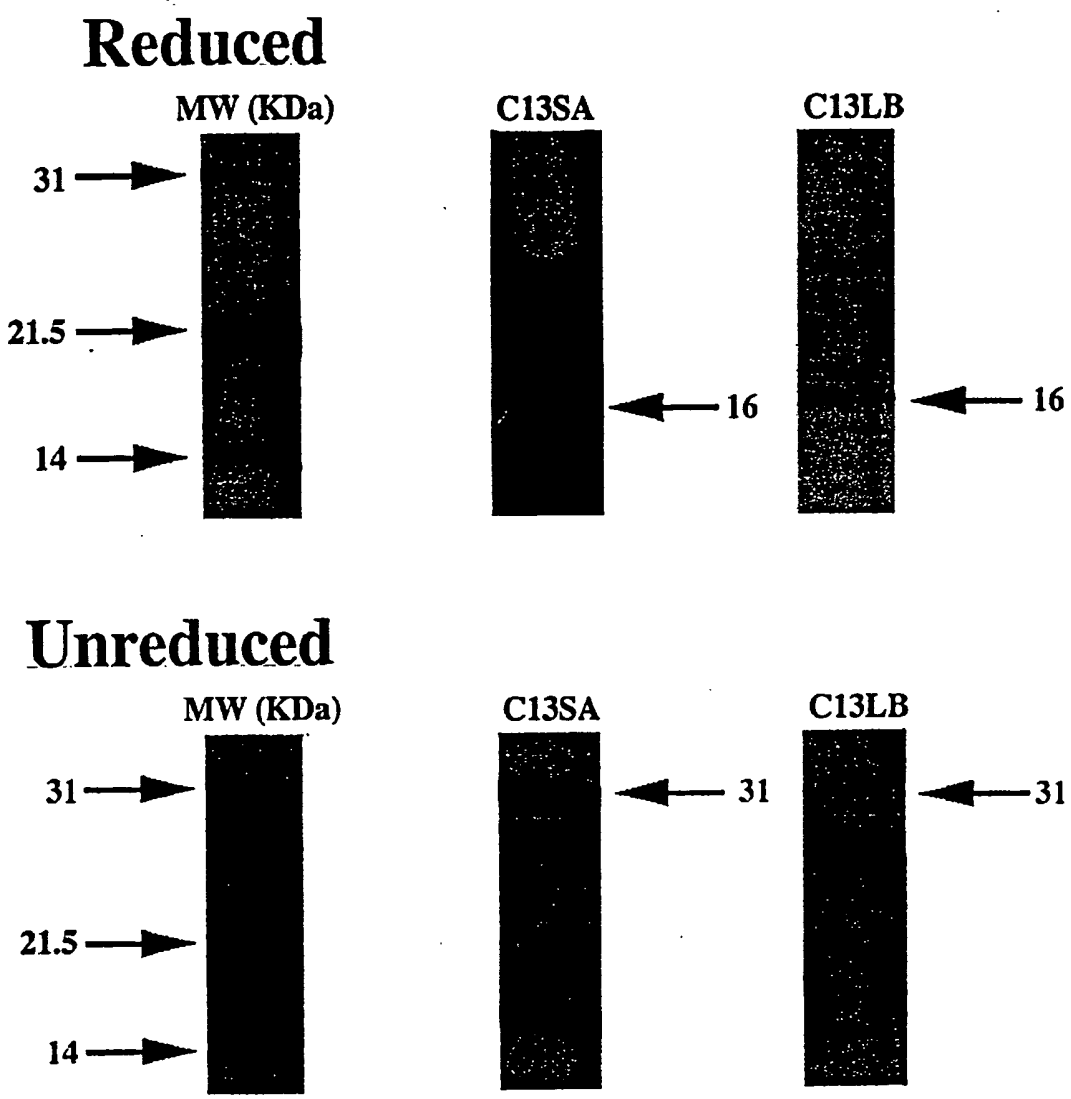


FIGURE 9

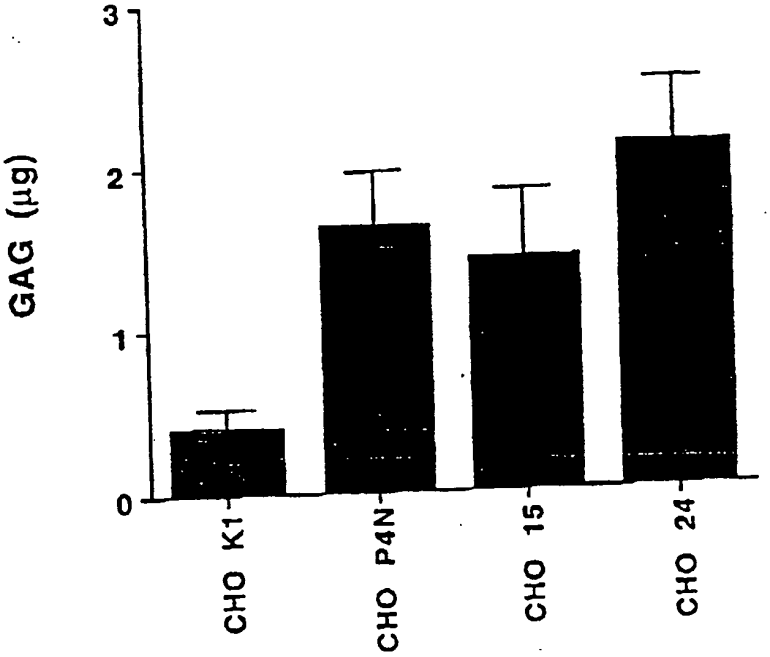


FIGURE 10

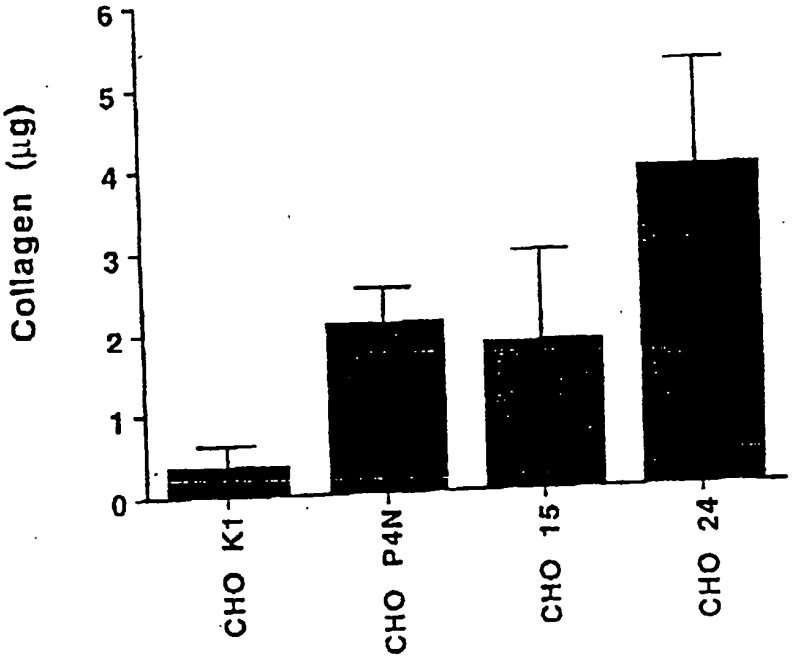
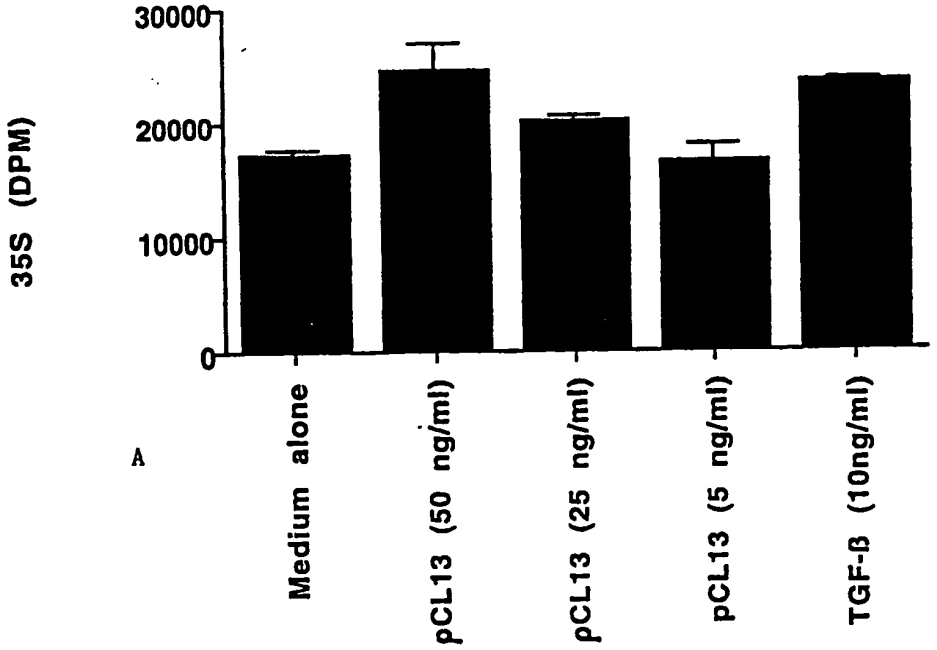
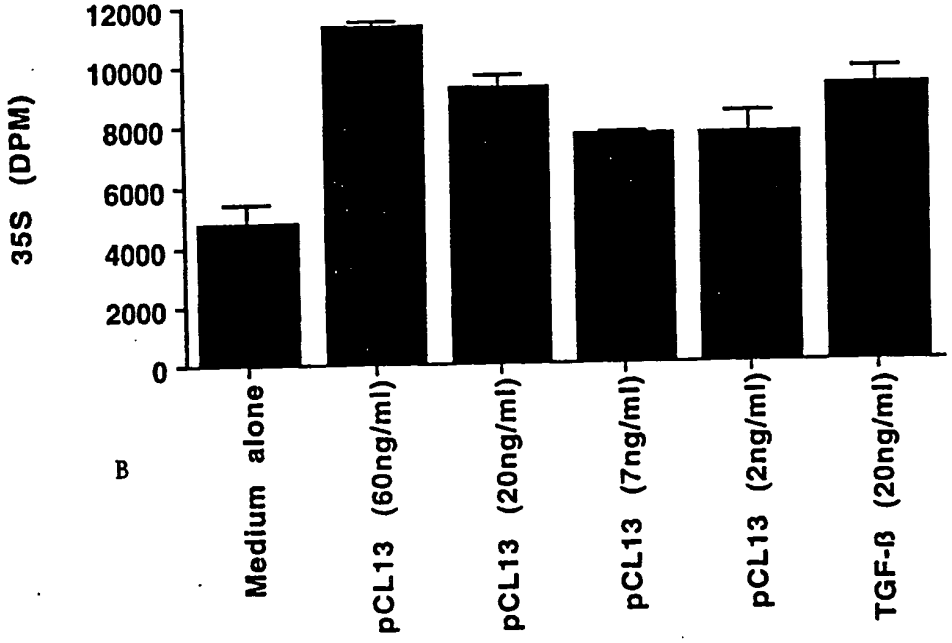


FIGURE 11


30000
20000
10000
0
35S (DPM)
Medium alone
pCL13 (50 ng/ml)
pCL13 (25 ng/ml)
pCL13 (5 ng/ml)
TGF-ß (10ng/ml)

A


12000
10000
8000
6000
4000
2000
0
35S (DPM)
Medium alone
pCL13 (60ng/ml)
pCL13 (20ng/ml)
pCL13 (7ng/ml)
pCL13 (2ng/ml)
TGF-ß (20ng/ml)

B

FIGURE 12

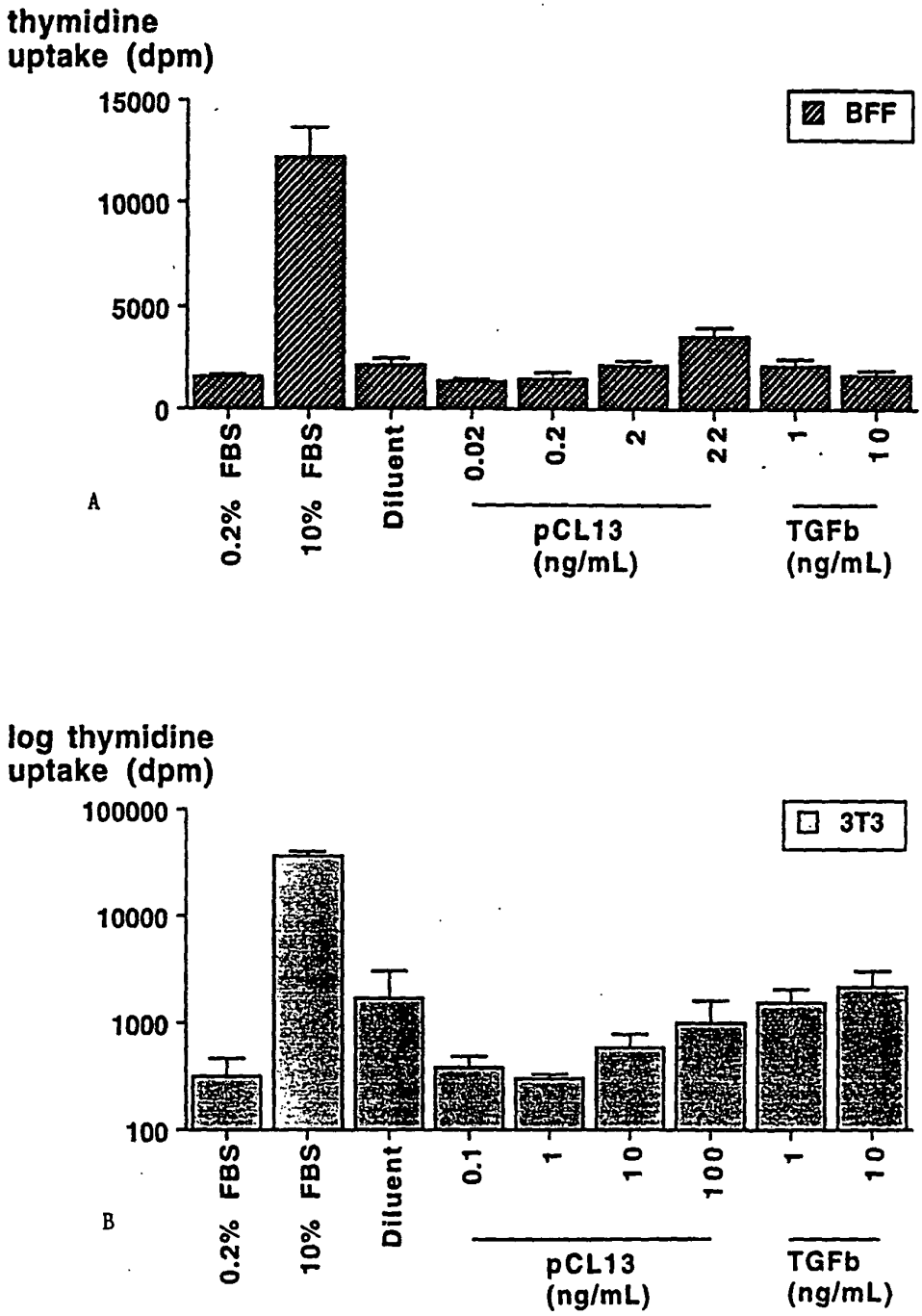

thymidine uptake (dpm)
15000
10000
5000
0
BFF
0.2% FBS
10% FBS
Diluent
0.02
0.2
2
22
1
10
A
pCL13 (ng/mL)
TGFb (ng/mL)
log thymidine uptake (dpm)
100000
10000
1000
100
3T3
0.2% FBS
10% FBS
Diluent
0.1
1
10
100
1
10
B
pCL13 (ng/mL)
TGFb (ng/mL)

FIGURE 14

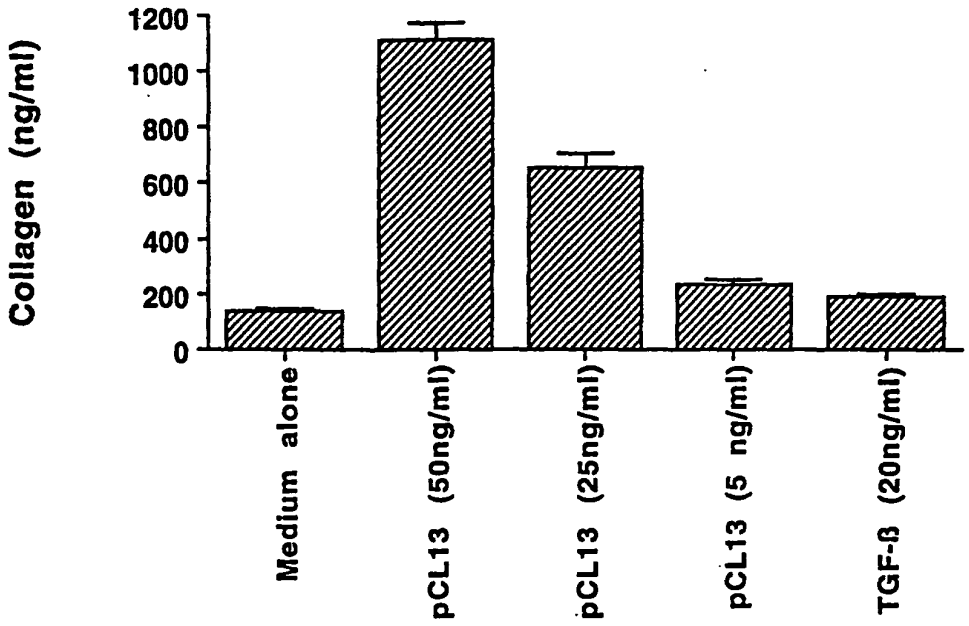
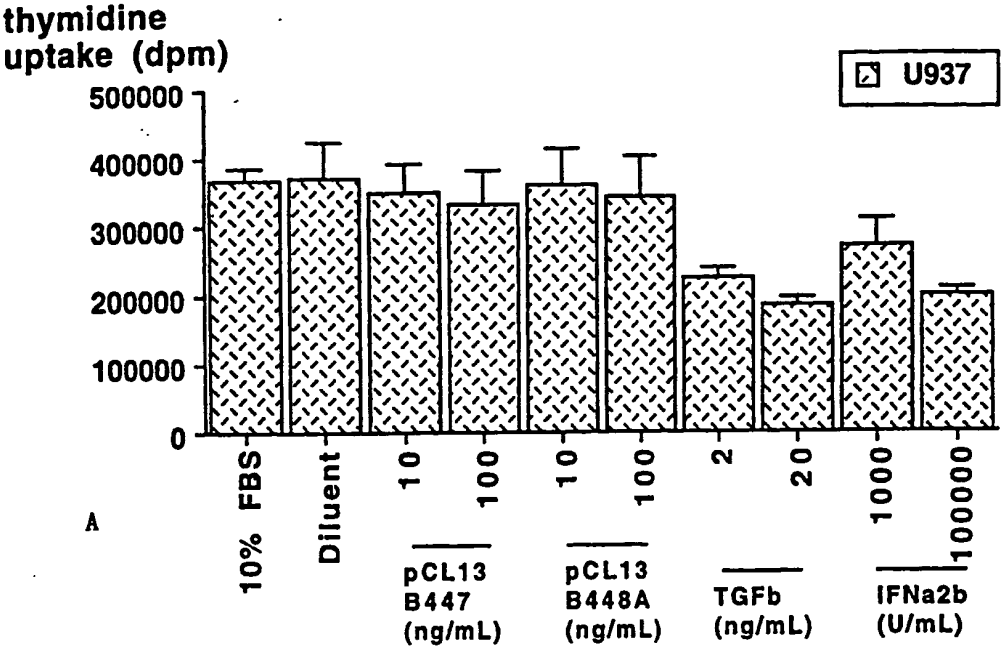

Collagen (ng/ml)
1200
1000
800
600
400
200
0
Medium alone
pCL13 (50ng/ml)
pCL13 (25ng/ml)
pCL13 (5 ng/ml)
TGF-β (20ng/ml)

FIGURE 13


thymidine uptake (dpm)
U937
500000
400000
300000
200000
100000
0
10% FBS
Diluent
10
100
10
100
2
20
1000
100000
pCL13 B447 (ng/mL)
pCL13 B448A (ng/mL)
TGFb (ng/mL)
IFNa2b (U/mL)
A

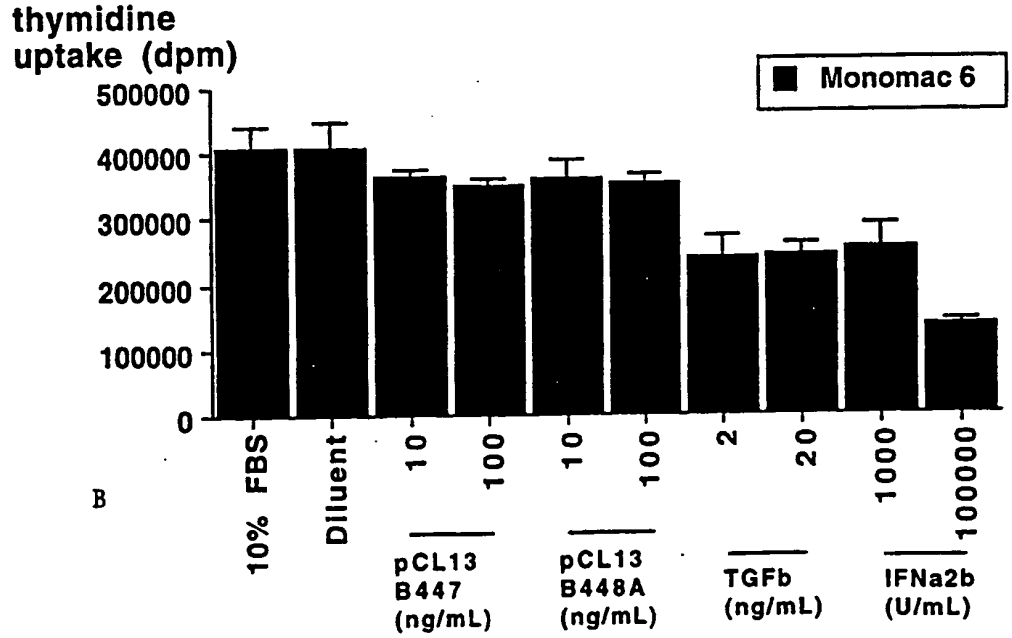
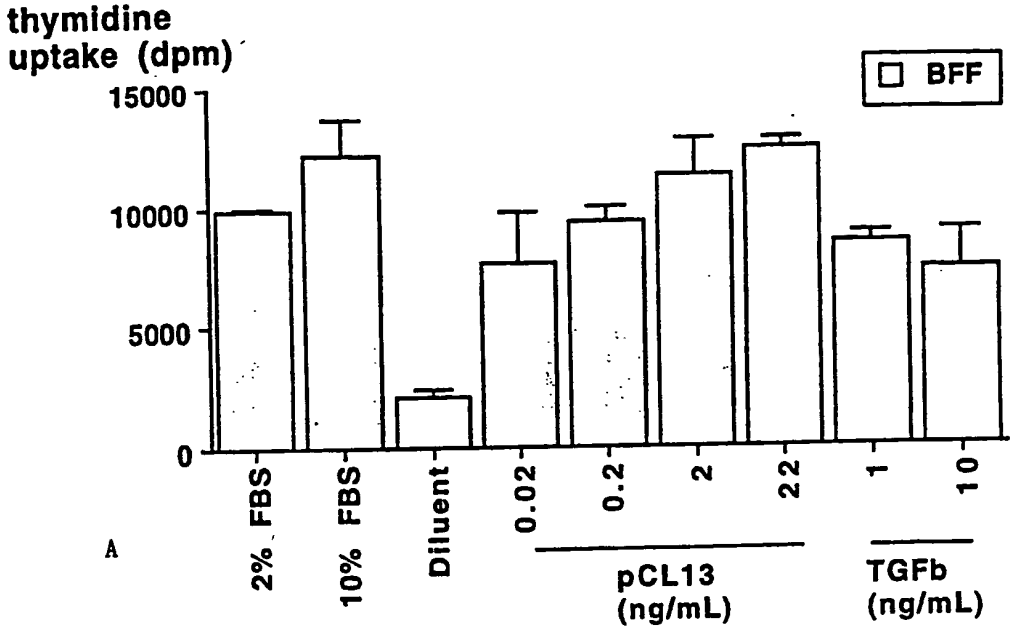

thymidine uptake (dpm)
Monomac 6
500000
400000
300000
200000
100000
0
10% FBS
Diluent
10
100
10
100
2
20
1000
100000
pCL13 B447 (ng/mL)
pCL13 B448A (ng/mL)
TGFb (ng/mL)
IFNa2b (U/mL)
B

FIGURE 16


thymidine uptake (dpm)
BFF
15000
10000
5000
0
2% FBS
10% FBS
Diluent
0.02
0.2
2
22
1
10
pCL13 (ng/mL)
TGFb (ng/mL)
A

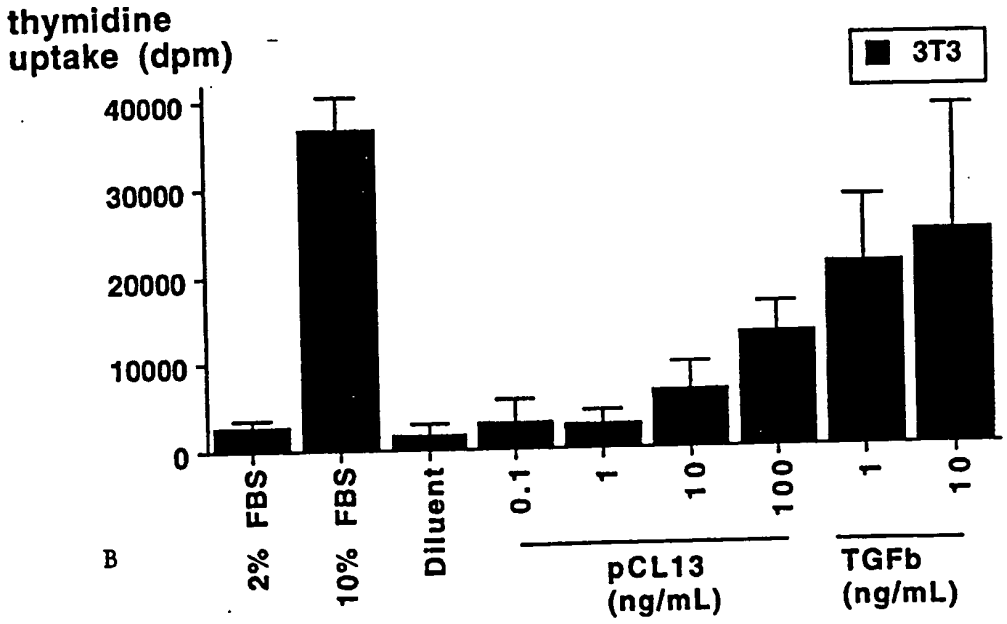

thymidine uptake (dpm)
3T3
40000
30000
20000
10000
0
2% FBS
10% FBS
Diluent
0.1
1
10
100
1
10
pCL13 (ng/mL)
TGFb (ng/mL)
B

FIGURE 15

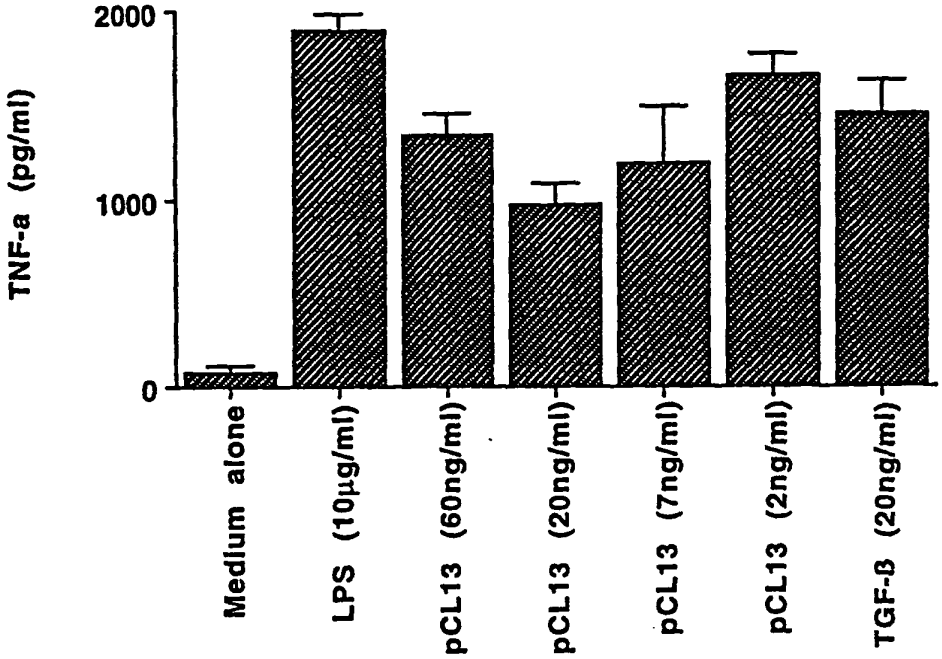

TNF-a (pg/ml)
2000
1000
0
Medium alone
LPS (10µg/ml)
pCL13 (60ng/ml)
pCL13 (20ng/ml)
pCL13 (7ng/ml)
pCL13 (2ng/ml)
TGF-ß (20ng/ml)

FIGURE 17

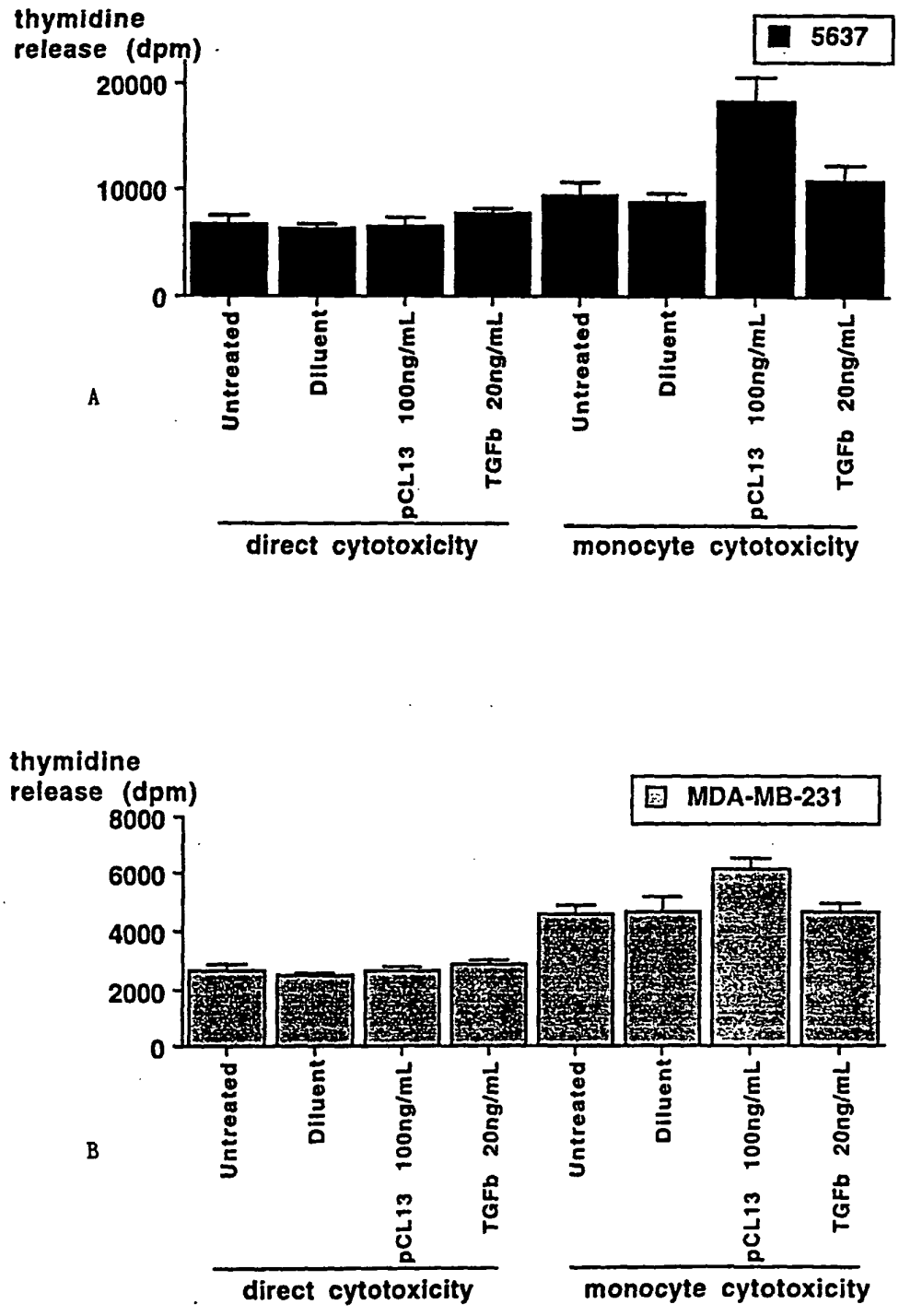

thymidine release (dpm)
5637
20000
10000
0
Untreated
Diluent
pCL13 100ng/mL
TGFb 20ng/mL
Untreated
Diluent
pCL13 100ng/mL
TGFb 20ng/mL
A
direct cytotoxicity
monocyte cytotoxicity
thymidine release (dpm)
MDA-MB-231
8000
6000
4000
2000
0
Untreated
Diluent
pCL13 100ng/mL
TGFb 20ng/mL
Untreated
Diluent
pCL13 100ng/mL
TGFb 20ng/mL
B
direct cytotoxicity
monocyte cytotoxicity

FIGURE 18

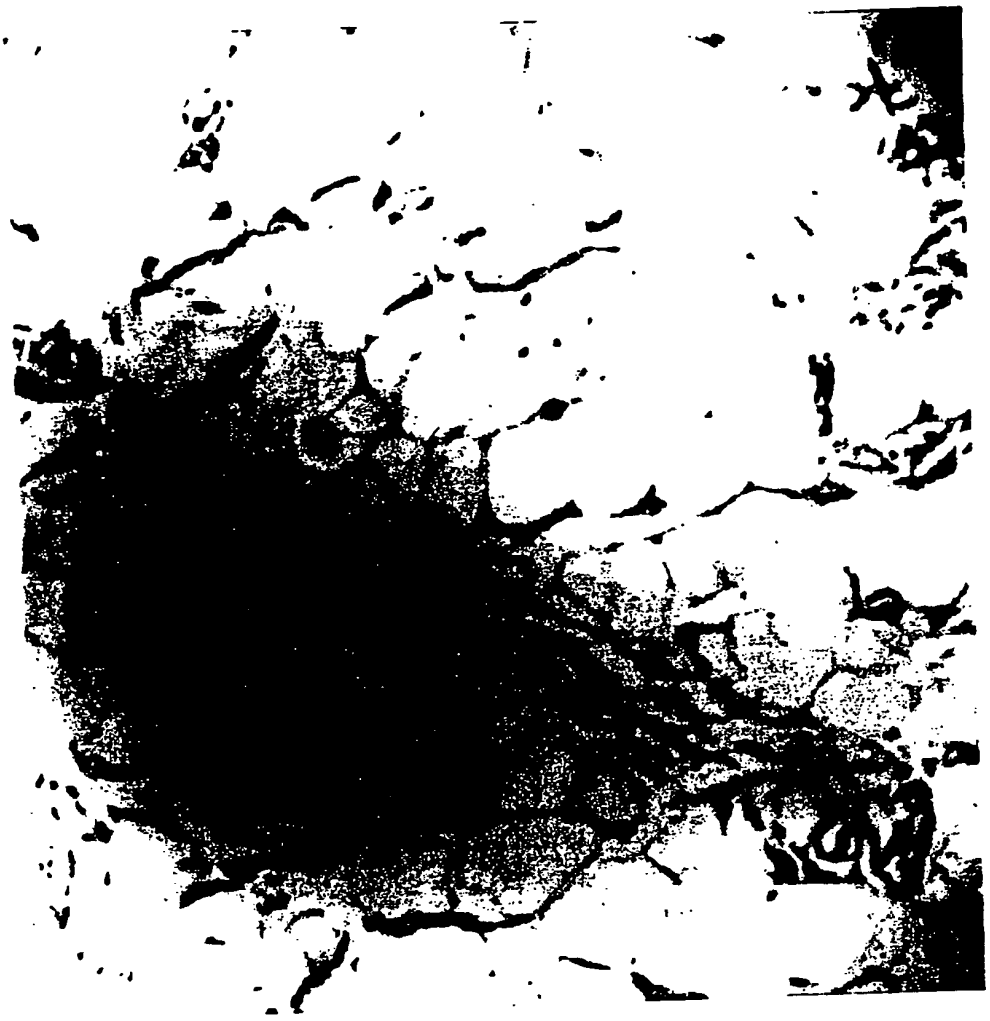


FIGURE 19B

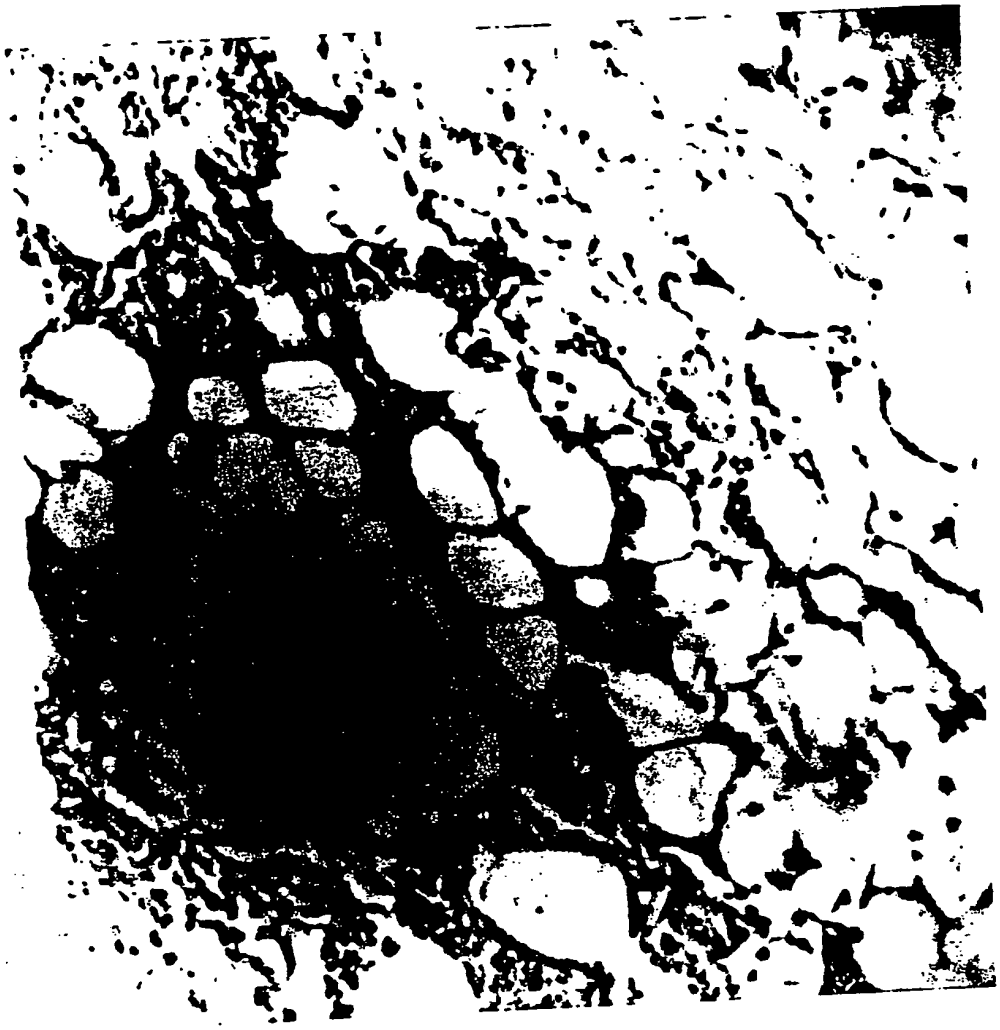


FIGURE 19A

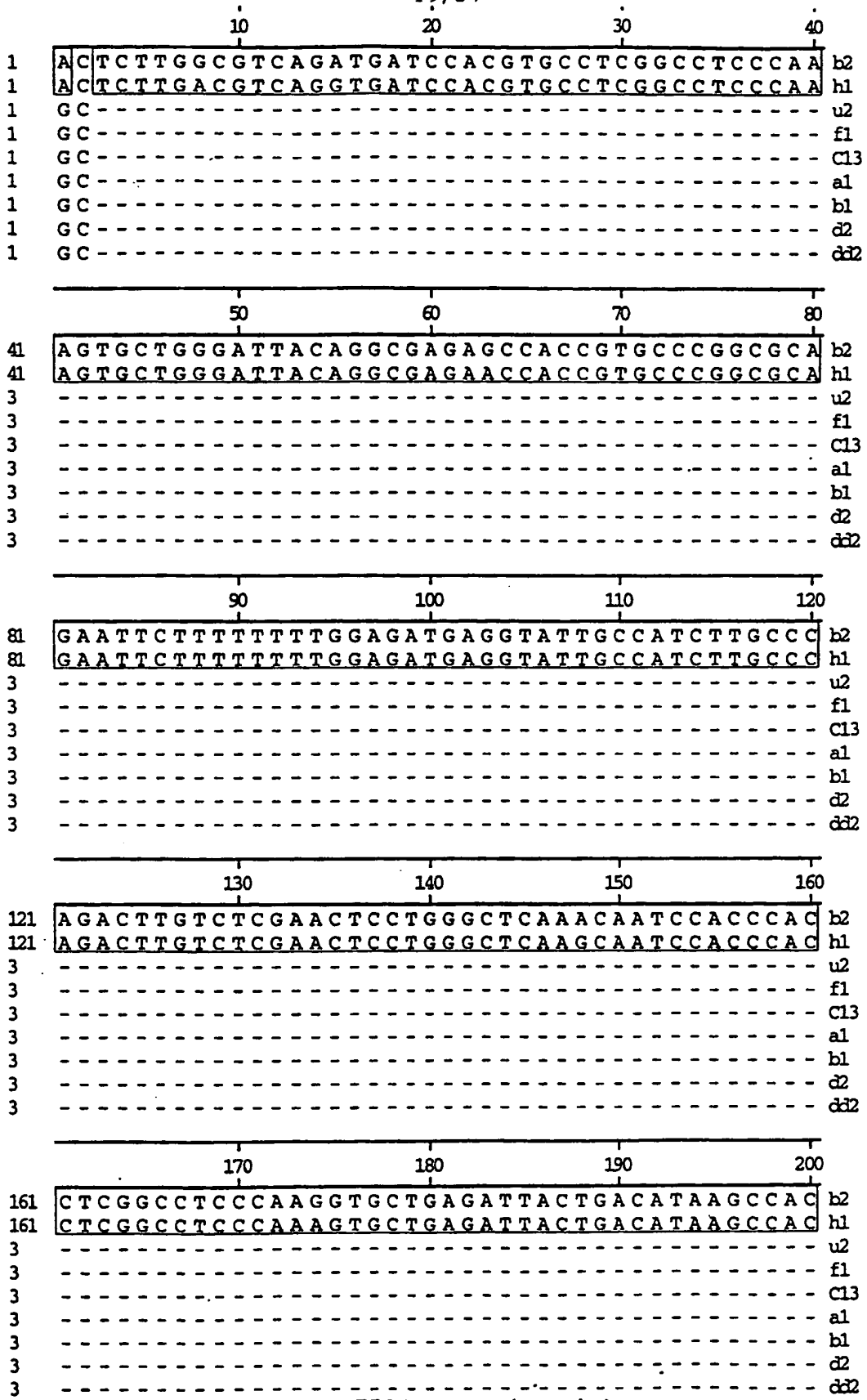


FIGURE 20A (Cont'd)

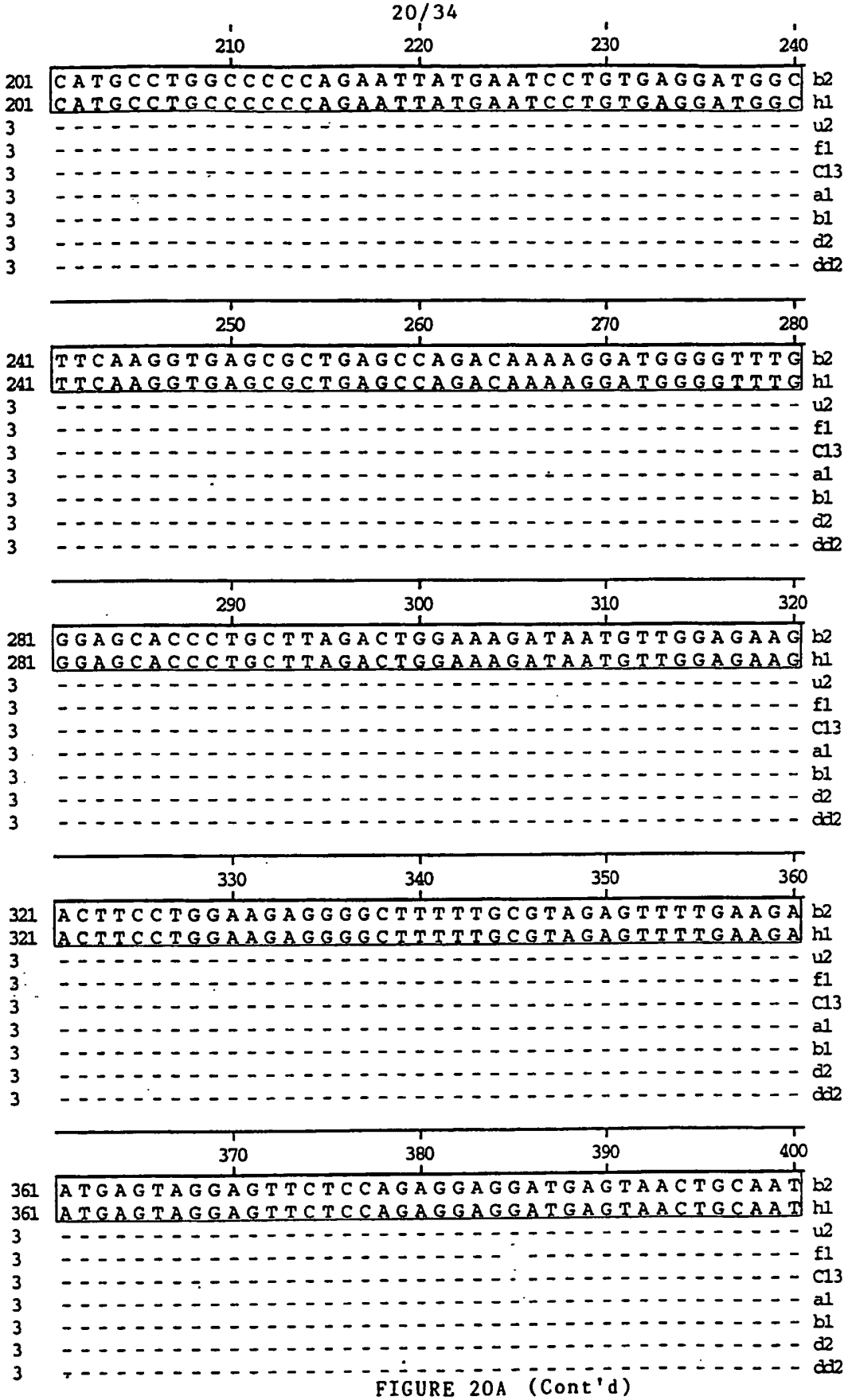


FIGURE 20A (Cont'd)

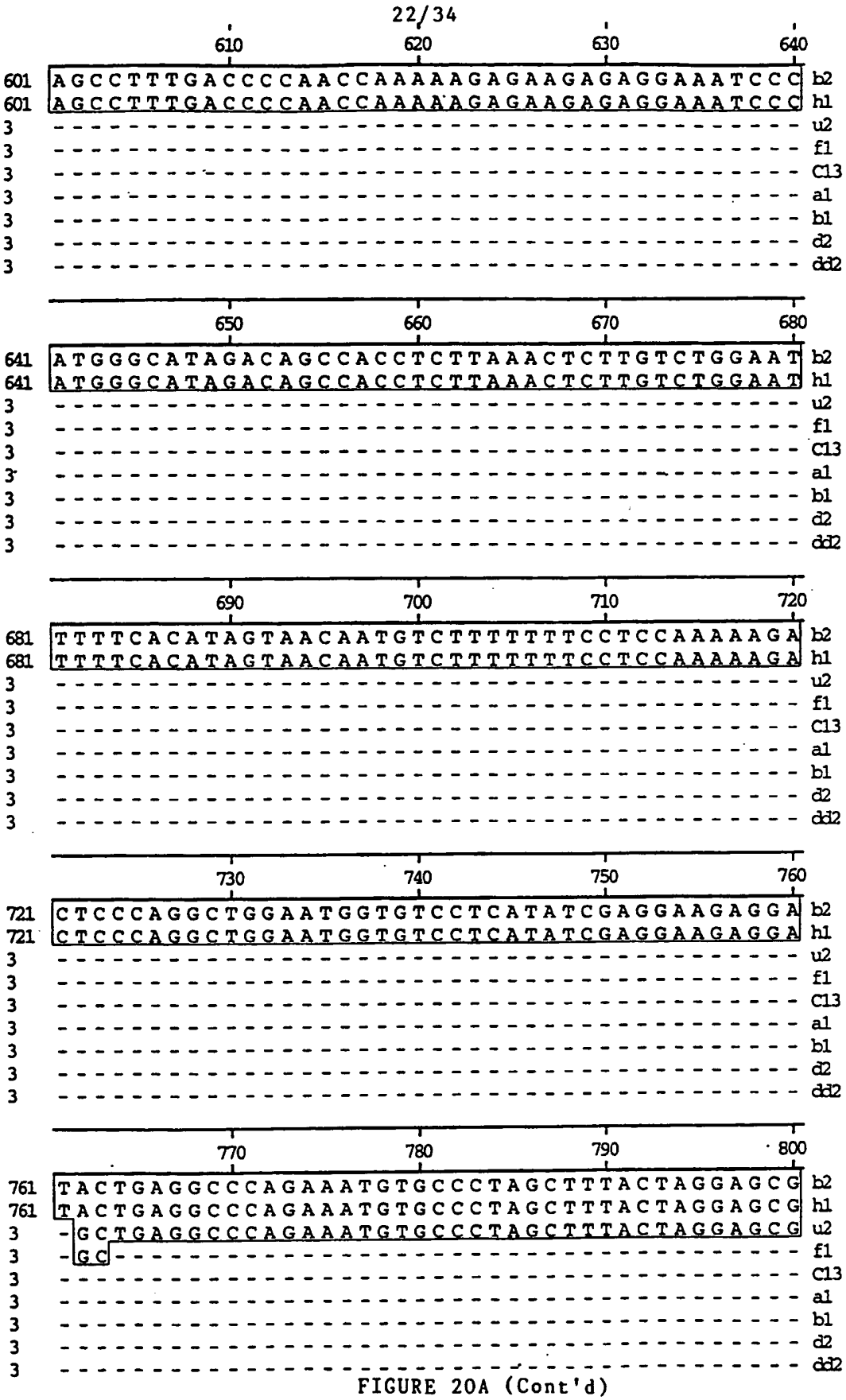


FIGURE 20A (Cont'd)

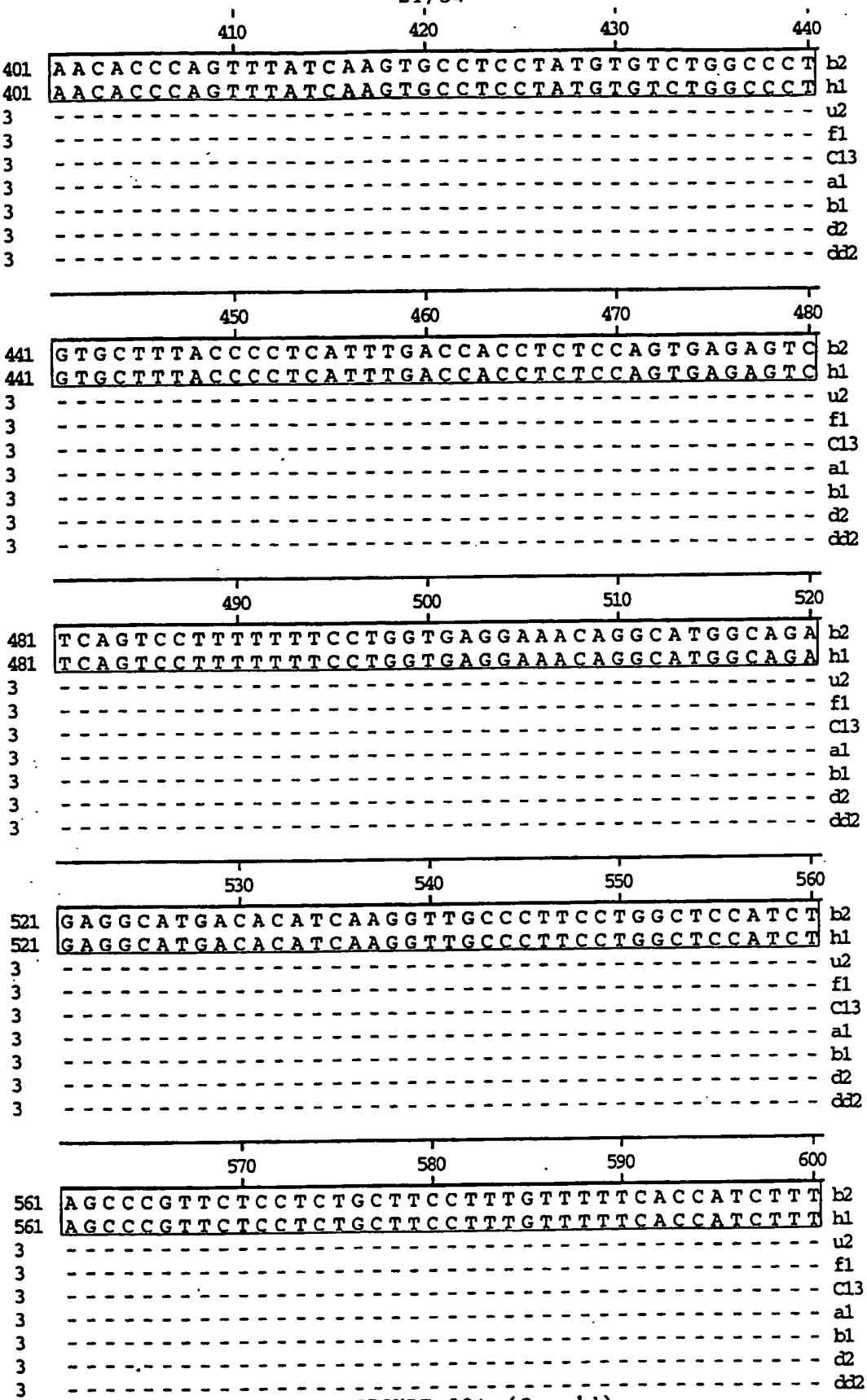


FIGURE 20A (Cont'd)

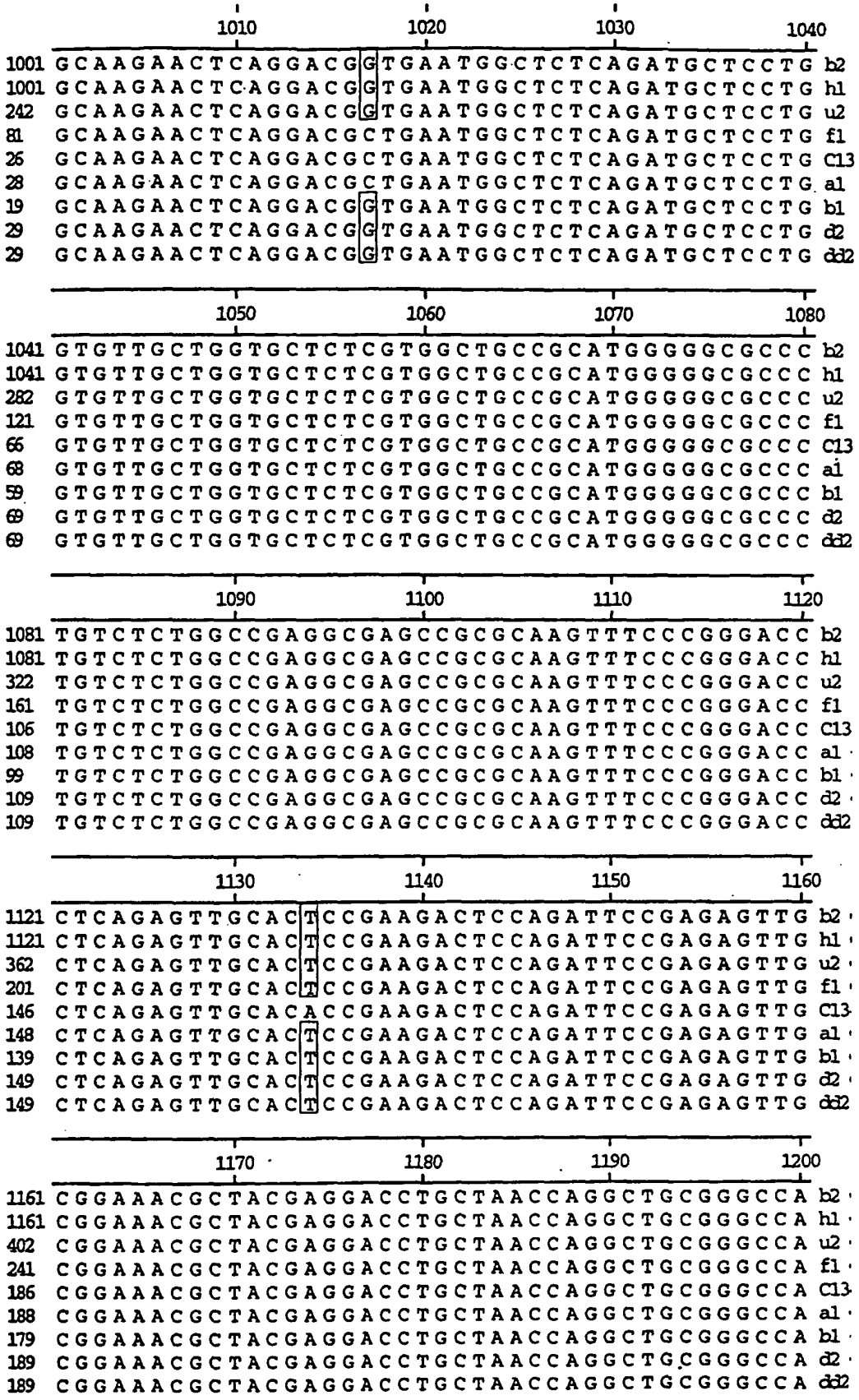


FIGURE 20A (Cont'd)

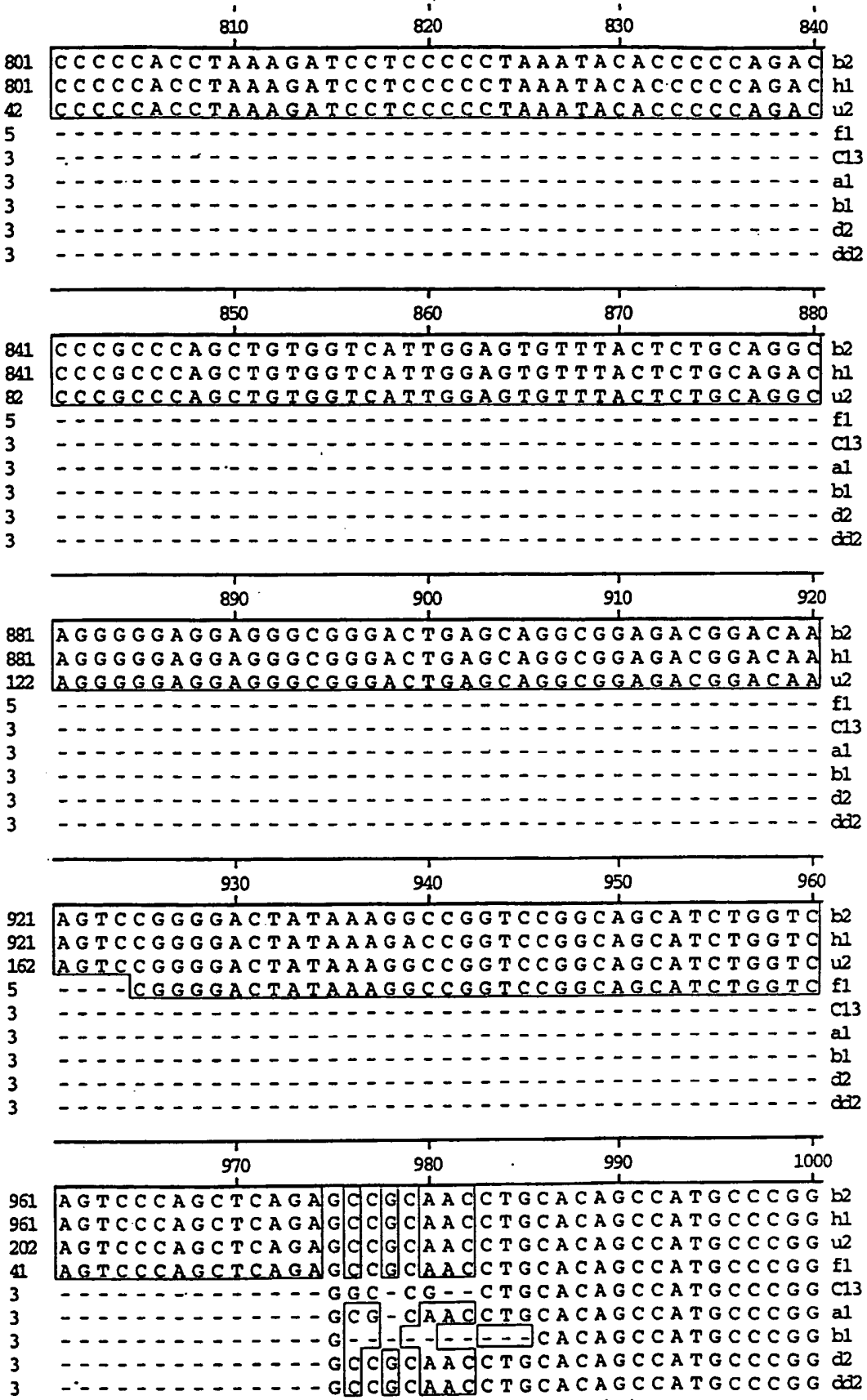


FIGURE 20A (Cont'd)

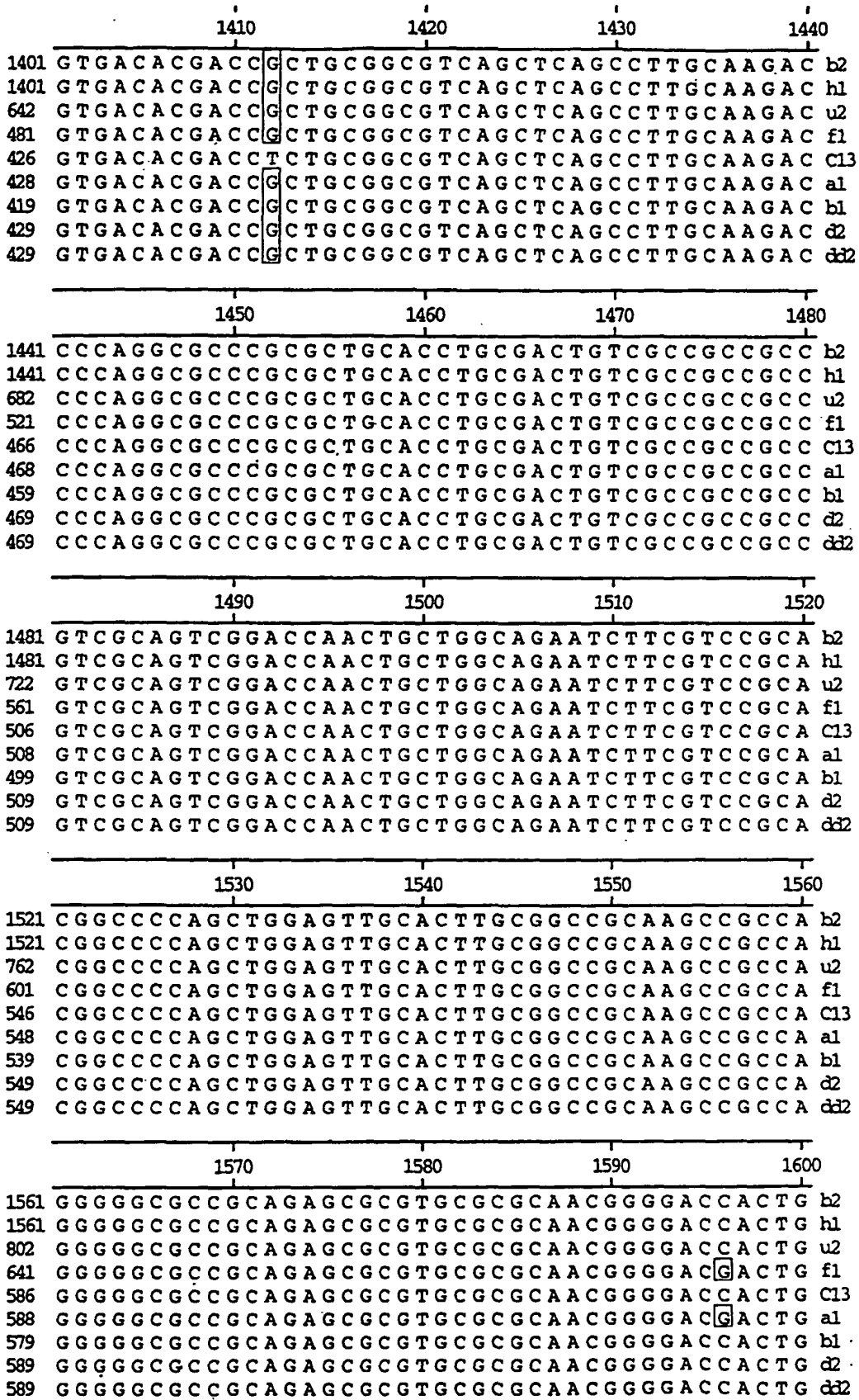


FIGURE 20A (Cont'd)

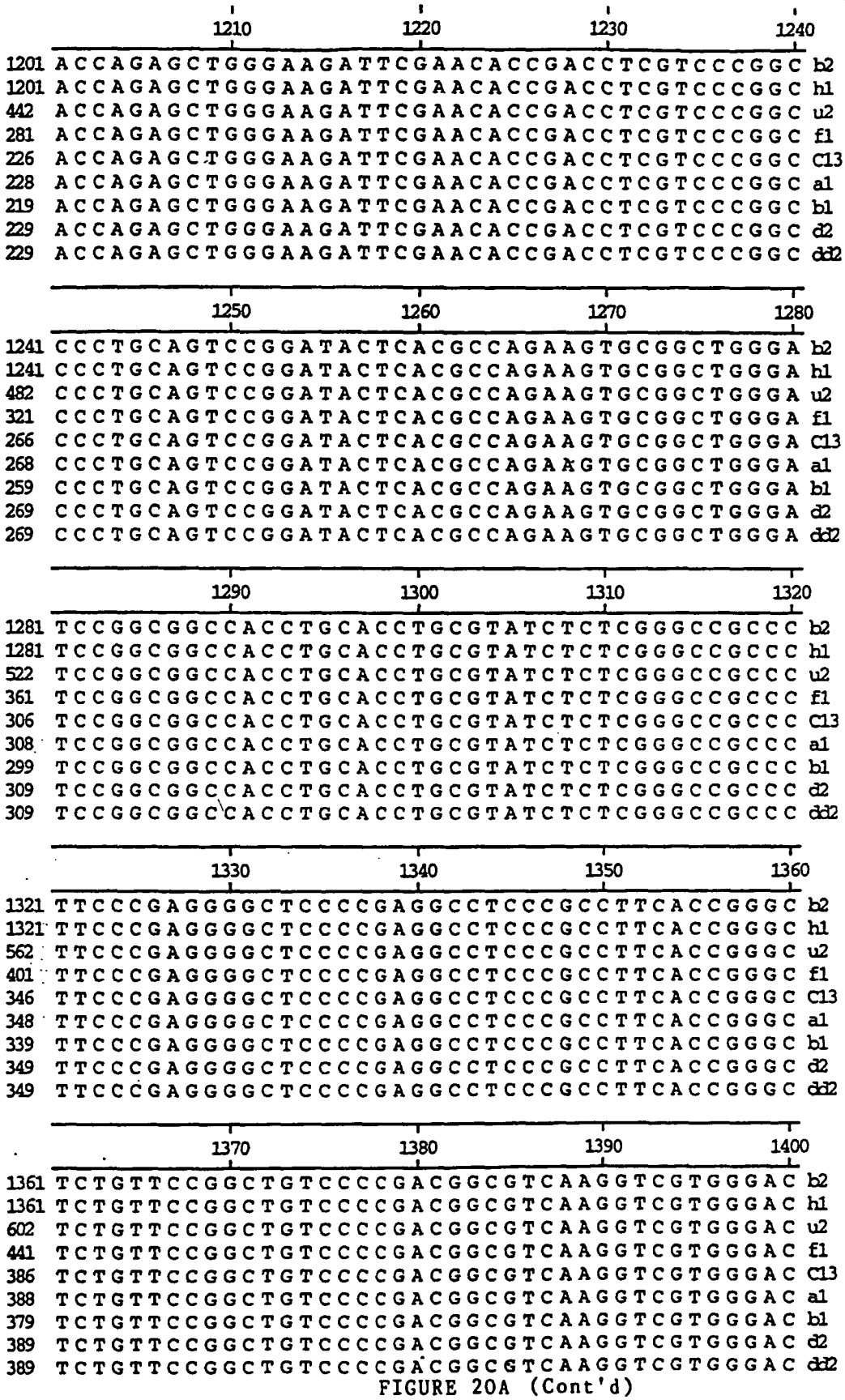


FIGURE 20A (Cont'd)

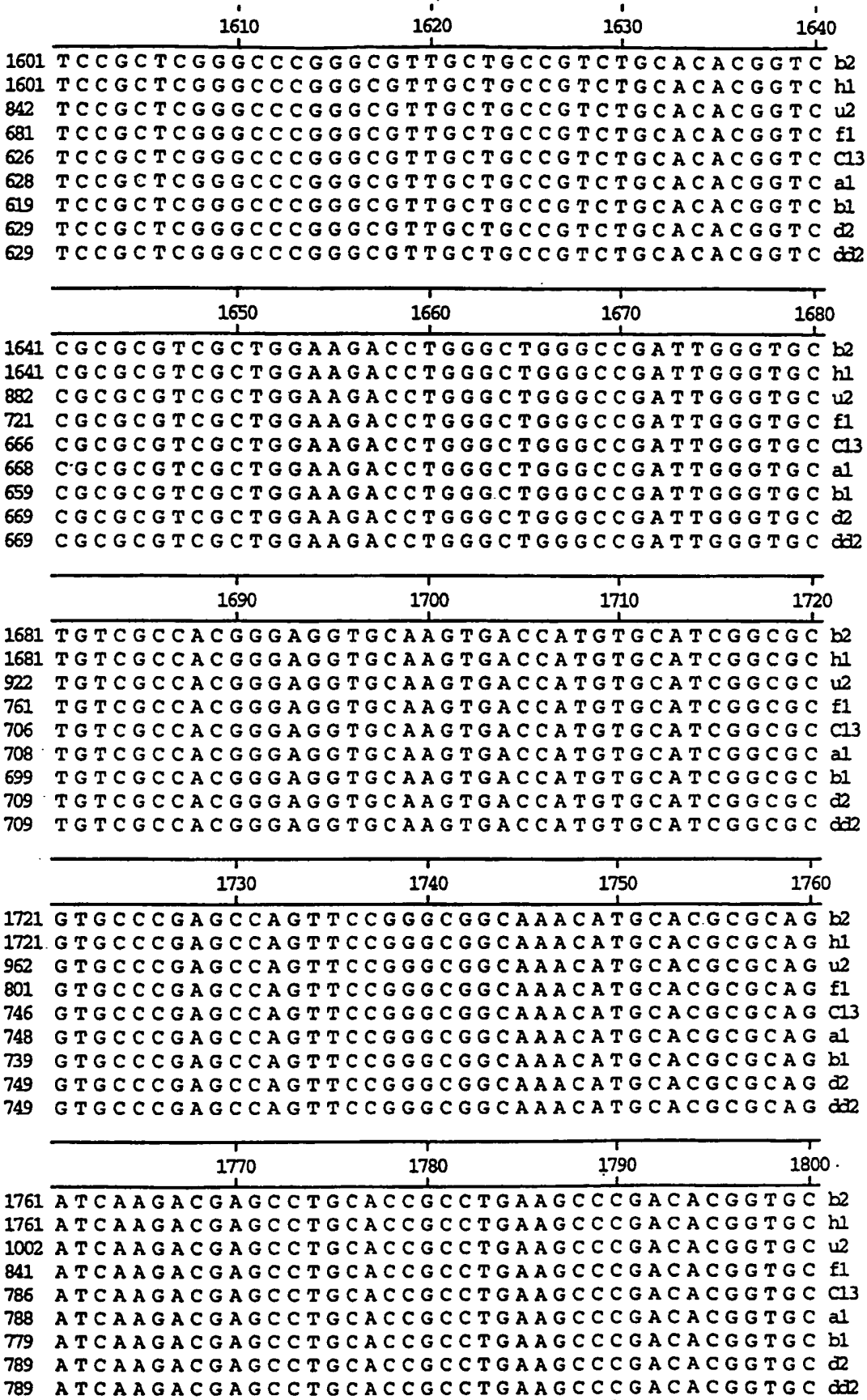


FIGURE 20A (Cont'd)

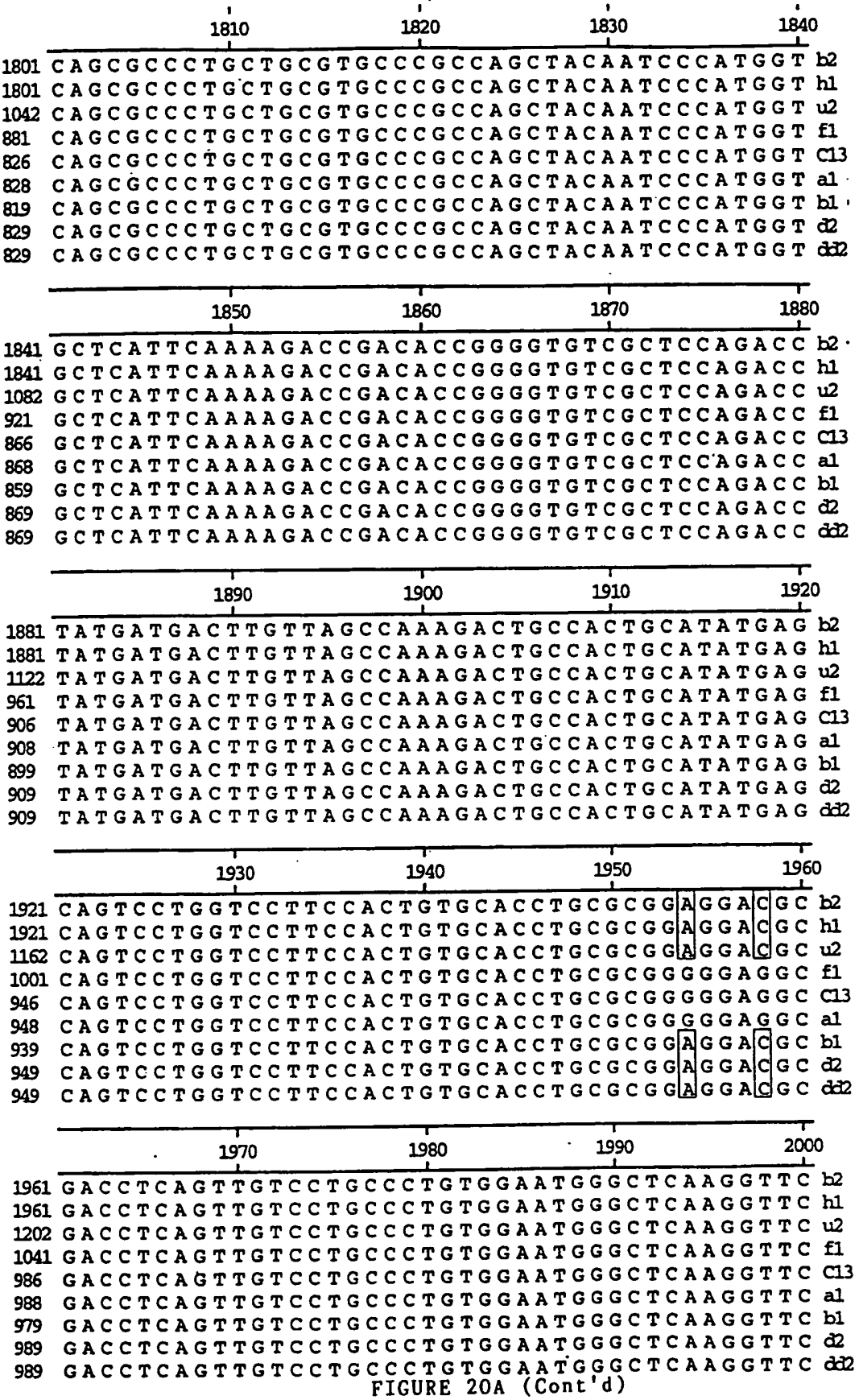


FIGURE 20A (Cont'd)

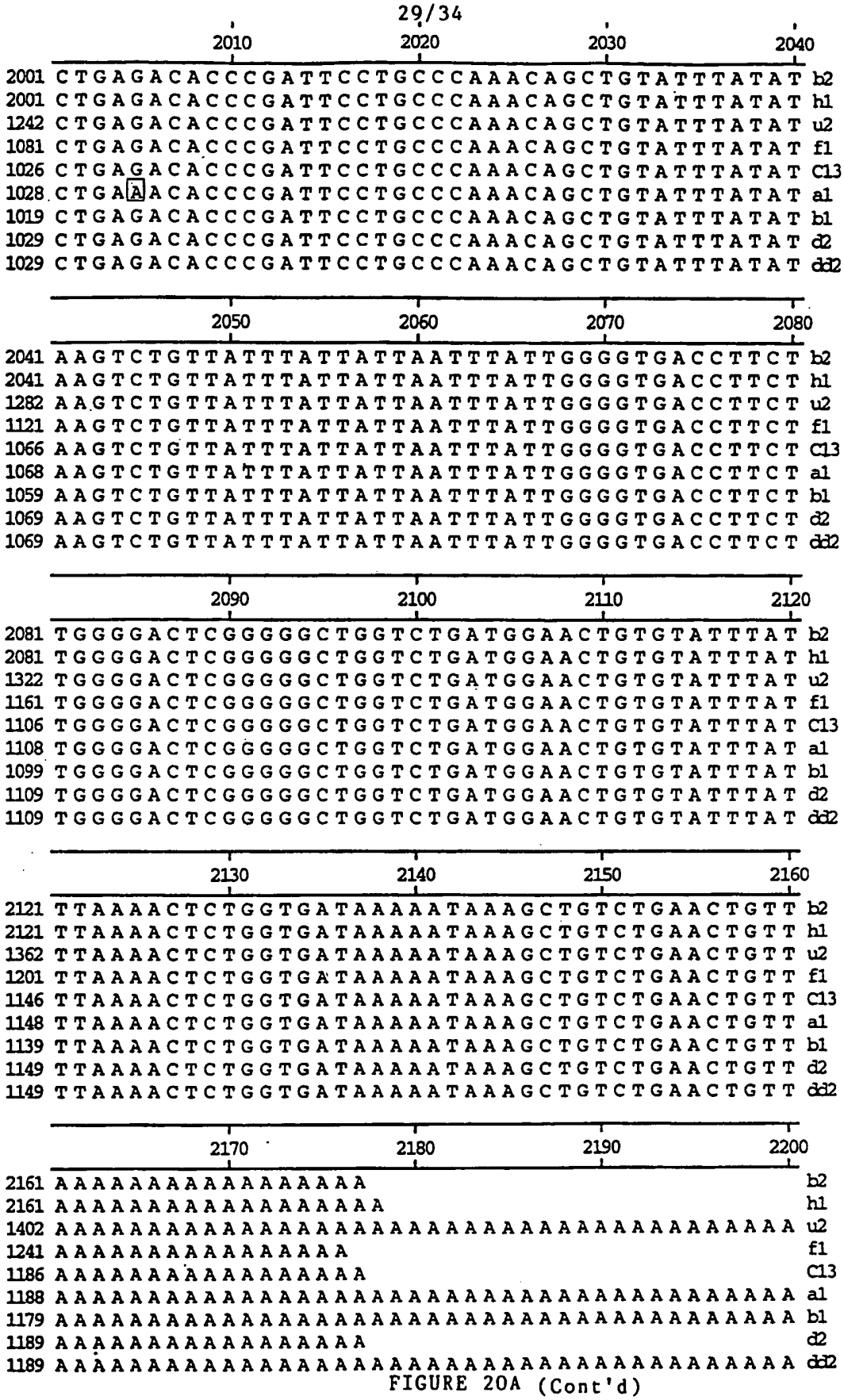


FIGURE 20A (Cont'd)

```
                    2210          2220          2230
                     |             |             |
2177                                                                  b2
2178                                                                  h1
1442 AAAAAAAAAAAAAAA                                                  u2
1256                                                                  f1
1202                                                                  C13
1228 AAAAAAAAAAA                                                      a1
1219 AAAAAAAAAAAAAAAAAAAAANAAAAANAAAAAAA                              b1
1205                                                                  d2
1229 AAAAAAAAAAAAAAAAAAAAAAAAAA                                       dd2
```

Decoration 'Decoration #1': Box residues that differ from C13dnaseq.def.

FIGURE 20A (Cont'd)

```
                    10        20        30        40
1   MPGQELRTLNGSQMLLVLLVLSWLPHGGALSLAEASRASF C13
1   MPGQELRTLNGSQMLLVLLVLSWLPHGGALSLAEASRASF a1
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF b1
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF b2
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF d2
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF dd2
1   MPGQELRTLNGSQMLLVLLVLSWLPHGGALSLAEASRASF f1
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF h1
1   MPGQELRTVNGSQMLLVLLVLSWLPHGGALSLAEASRASF u2

                    50        60        70        80
41  PGPSELHTEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL C13
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL a1
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL b1
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL b2
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL d2
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL dd2
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL f1
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL h1
41  PGPSELHSEDSRFRELRKRYEDLLTRLRANQSWEDSNTDL u2

                    90        100       110       120
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL C13
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL a1
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL b1
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL b2
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL d2
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL dd2
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL f1
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL h1
81  VPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRL u2

                    130       140       150       160
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS C13
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS a1
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS b1
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS b2
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS d2
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS dd2
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS f1
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS h1
121 HRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLS u2

                    170       180       190       200
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG C13
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG a1
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG b1
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG b2
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG d2
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG dd2
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG f1
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG h1
161 PPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNG u2
```

FIGURE 20B

```
                  210       220       230       240
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC C13
201 DDCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC a1
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC b1
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC b2
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC d2
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC dd2
201 DDCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC f1
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC h1
201 DHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTMC u2

                  250       260       270       280
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN C13
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN a1
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN b1
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN b2
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN d2
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN dd2
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN f1
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN h1
241 IGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYN u2

                  290       300
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. C13
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. a1
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. b1
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. b2
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. d2
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. dd2
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. f1
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. h1
281 PMVLIQKTDTGVSLQTYDDLLAKDCHCI. u2
```

Decoration 'Decoration #1': Box residues that differ from C13protseq.def.

FIGURE 20B (Cont'd)

C13SA/5H

NUCLEOTIDE SEQUENCE

```
        |  10     |  20     |  30     |  40     |  50     |  60     |  70     |  80
  1 GCTAGCGCCA TGGATTACTA CAGAAAATAT GCAGCTATCT  TTCTGGTCAC ATTGTCGGTG TTTCTGCATG TTCTCCATTC 80
 81 CGCTCCTGAT GAATTCCACC ACCACCACCA CCTGGTGCCC CGCGGCTCCG ACTACAAGGA CGACGACGAC AAGCTCCGCG 160
161 CCTCCGTGGC GCGCAACGGG GACCACTGTC CGCTCGGGCC CGGGCGTTGC TGCCGTCTGC ACACGGTCCG CGCGTCGCTG 240
241 GAAGACCTGG GCTGGGCCGA TTGGGTGCTG TCGCCACGGG AGGTGCAAGT GACCATGTGC ATCGGCGCGT GCCCGAGCCA 320
321 GTTCCGGGCG GCAAACATGC ACGCGCAGAT CAAGACGAGC CTGCACCGCC TGAAGCCCGA CACGGTGCCA GCGCCCTGCT 400
401 GCGTGCCCGC CAGCTACAAT CCCATGGTGC TCATTCAAAA GACCGACACC GGGGTGTCGC TCCAGACCTA TGATGACTTG 480
481 TTAGCCAAAG ACTGCCACTG CATATGACTC GAG                                                  513
        |  10     |  20     |  30     |  40     |  50     |  60     |  70     |  80
```

TRANSLATED PROTEIN SEQUENCE

```
        |  10     |  20     |  30     |  40     |  50     |  60     |  70     |  80
  1 LEKREHHHHH LVPRGSDYKD DDDKLRASVA RNGDHCPLGP  GRCCRLHTVR ASLEDLGWAD WVLSPREVQV TMCIGACPSQ 80

 81 FRAANMHAQI KTSLHRLKPD TVPAPCCVPA SYNPMVLIQK  TDTGVSLQTY DDLLAKDCHC I •                 142
        |  10     |  20     |  30     |  40     |  50     |  60     |  70     |  80
```

underline = FLAG epitope
═══ = PKA site
↓ = yeast signal sequence cleavage site amino acid 5
• = stop codon
┌──┐ = motif of 5 HIS residues for binding to metal chelate column
coding region for bioactive part of clone 13 commences with amino acid 30
LVPRGS = this sequence from amino acids 11-16 represents thrombin cleavage site

FIGURE 21

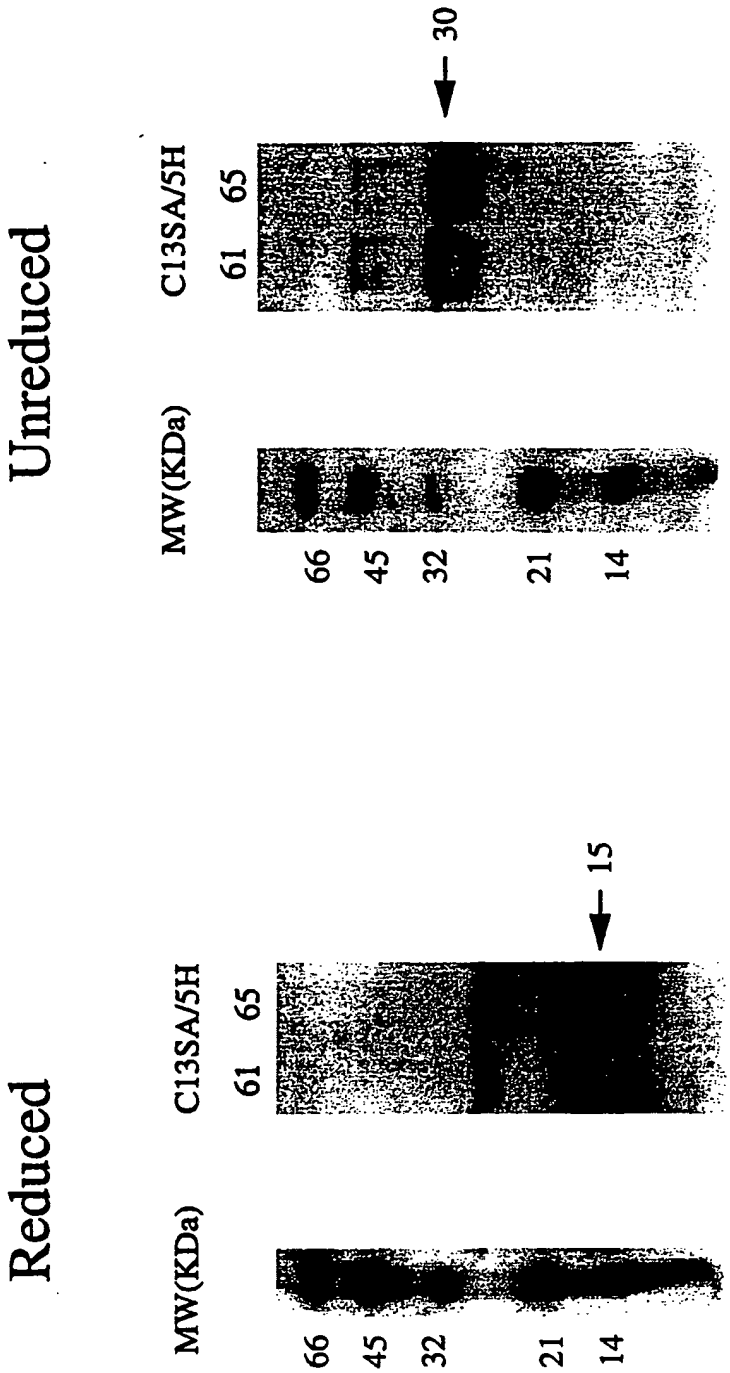

Unreduced
30
C13SA/5H
61 65
MW(KDa)
66 45 32 21 14
Reduced
15
C13SA/5H
61 65
MW(KDa)
66 45 32 21 14

FIGURE 22